FORM PTO-1390 -(REV 10-95)

U.S. P. ARTIM LIT OF COMMERCE PATENT AND TRADEMARK OFFICE ATTORNEY'S DOCKET NUMBER

## TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371

MERCK 2332

U.S. APPLICATION NO. (If known, see 37 CFR §1.5)

10/009500

	CONCERNING	JATIERIO CIDERIOS CISICI 307	10/00/200
INTERNA	ATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/	EP00/05181	6 JUNE 2000	12 JUNE 1999
HYALI	FINVENTION URONIDASE FROM THI UCTION	E <i>HIRUDINARIA MANILLENSIS</i> , ISOLATION, PUR	IFICATION AND RECOMBINANT METHOD OF
APPLICA	ANT(S) FOR DO/EO/US		
KOR	DOWICZ, Maria, et	t al.	ŝ
Applic	ant herewith submits to t	the United States Designated/Elected Office (DO/EC	O/US) the following items and other information:
1.	This is a FIRST submi	ssion of items concerning a filing under 35 U.S.C. §3	71.
2.	This is a SECOND or S	SUBSEQUENT submission of items concerning a fili	ing under 35 U.S.C. §371.
3.	This express request to expiration of the applic	begin national examination procedures (35 U.S.C. §3 able time limit set in 35 U.S.C. §371(b) and PCT Arti	71(f)) at any time rather than delay examination until the cles 22 and 39(1).
4.	A proper Demand for I	nternational Preliminary Examination was made by th	e 19th month from the earliest claimed priority date.
5.	A copy of the Internation	onal Application as filed (35 U.S.C. §371(c)(2))	
	a. $\square$ is transmitted	herewith (required only if not transmitted by the Inte	rnational Bureau).
	b. has been tran	smitted by the International Bureau.	7
		ed, as the application was filed in the United States Re	
6. 🗆		ernational Application into English (35 U.S.C. §371(c	
7.	4	ims of the International Application under PCT Articl	
		ed herewith (required only if not transmitted by the In-	ternational Bureau).
	4	ansmitted by the International Bureau.	
	_	n made; however, the time limit for making such amer	ndments has NOT expired.
	•	n made and will not be made.	G (2271/C)/2))
8. L	_	nendments to the claims under PCT Article 19 (35 U.S	.C. §3/1(c)(3)).
9.		of the inventor(s) (35 U.S.C. §371(c)(4)).	. 1 DOT AND 1 26 (25 H 2 C 2271/-)(5))
10. 🗆		nexes to the International Preliminary Examination Red document(s) or information included:	eport under PCT Article 36 (35 U.S.C. §3/1(c)(5)).
items 1½: □		usure Statement under 37 C.F.R. §§1.97 and 1.98.	
12.		ent for recording. A separate cover sheet in compliance	ce with 37 C.F.R. §§3.28 and 3.31 is included.
	_		55 min 57 Civing \$35,250 and 5.57 to moradon
13.	A FIRST preliminary a	amenament. EQUENT preliminary amendment.	
	•		
14.			
15.		attorney and/or address letter.	
] 16. <b>=</b>	Other items or informa	ation: Paper Copy of Sequence Listing	
1			
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J.S. APPLICA	TION NO. (if kno	wn, see 37 CFR §	1.5) 🍖 🔠	INTERNATIONAL APPLICATION NO	).	ATTORNEY'S DOCKET NUM	IBEK
	10/	<u> </u>	UU	PCT/EP00/05181		MERCK 2332	
7. 🛛 T	The following	fees are subm	itted:			CALCULATIONS	PTO USE ONLY
F	BASIC NATIO	ONAL FEE (	37 CFR §1.4	92 (a) (1) - (5)):			
S	Search Report	has been prep	ared by the El	PO or JPO	\$890.00		
I	nternational p	reliminary ex	amination fee	paid to USPTO (37 CFR §1.482	2) \$710.00		
l b	No internation out internation	al preliminary al search fee p	examination paid to USPTO	fee paid to USPTO (37 CFR §1 ) (37 CFR §1.445(a)(2))	.482) \$740.00		
ì	Neither internanternal se	ational prelimearch fee (37	inary examina CFR §1.445(a	tion fee (37 CFR §1.482) nor (2)) paid to USPTO	\$1040.00		
I a	International p and all claims	satisfied prov	isions of PCT	paid to USPTO (37 CFR §1.48 Article 33(2)-(4)	\$100.00		
		EN	TER APPI	ROPRIATE BASIC FI	EE AMOUNT =	\$890.00	
Surcharge months fro	of \$130.00 fo om the earliest	r furnishing tl claimed prio	ne oath or decl rity date (37 C	aration later than .F.R. §1.492(e)).	30		
CL	AIMS	NUMBE	R FILED	NUMBER EXTRA	RATE		
Total clain	ns	20	- 20 =	0	x \$ 18.00	\$0.00	
Independe	ent claims	3	- 3 =	0	x \$ 84.00	\$0.00	
MULTIPL	LE DEPENDE	NT CLAIM(S	S) (if applicabl	e)	+ \$ 280.00		
			TO	TAL OF ABOVE CAL	CULATIONS =	\$890.00	
Reduction	of 1/2 for fili	ng by small e	ntity, if applica	able. A Verified Small Entity S	tatement must also be		
					SUBTOTAL =	\$890.00	
Processing months fro	g fee of \$130.0 om the earlies	00 for furnish claimed prio	ing the English rity date (37 C	n translation later than 20 2.F.R. §1.492(f)).	30		
		<u> </u>		TOTAL NA	TIONAL FEE =	\$890.00	
Fee for re	cording the en	closed assign	ment (37 C.F.)	R. §1.21(h)). The assignment n . \$40.00 per property.	nust be accompanied b	>	
an approp	riate cover site	et (37 C.F.R.	993.26, 3.31)		S ENCLOSED =	\$890.00	
		.,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,				Amount to be refunded:	
						charged:	
a.	A check in	the amount o	f \$890.0	0 to cover the above fees	is enclosed.		
, b. 🗆	Please cha A duplicate	rge my Dep e copy of this	osit Account sheet is enclo	No. 13-3402 in the amount	of \$	to cover the above fees	5.
c.	The Comm	issioner is he	reby authorize	d to charge any additional fees	which may be required	, or credit any overpaym	ent to
* · ·	Deposit Ac			duplicate copy of this sheet is			
NO rev	TE: Where ive (37 C.F.	e an approj R. §1.137(a	oriate time l a) or (b)) m	imit under 37 C.F.R. §§1 ust be filed and granted to	.494 or 1.495 has no restore the applic	ot been met, a petiti ation to pending sta	on to tus.
SEND AL	L CORRESPO	NDENCE TO:	Customer Num	ber 23,599			
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	10 DECE	EMBER 20	001		27,969 REGISTRAT	ION NUMBER	
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nmo.				maga 2 of 2			(November

### IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: MERCK 2332

In re patent application of

KORDOWICZ, MARIA et al.

Serial No. 10/009,500

Filed: April 8, 2002

For: HYALURONIDASE FROM THE HIRUDINARIA MANILLENSIS, ISOLATION,

PURIFICATION AND RECOMBINANT METHOD OF PRODUCTION

# STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents Washington, D.C. 20231
Box SEQUENCE

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

- the submission, filed herewith in accordance with 37
   C.F.R. § 1.821(g), does not include new matter;
- 2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same.

Respectfully submitted,

HARBOR CONSULTING

Intellectual Property Services 1500A Lafayette Road Suite 262 Portsmouth, N.H. 800-318-3021

#### SEQUENCE LISTING

- <110 > KORDOWICZ, MARIA GUESSOW, DETLET HOFMANN, UWE PACUSZKA, TADEUSZ GARDAS, ANDRZEJ
- <120> HYALURONIDASE FROM THE HIRUDINARIA MANILLENSIS, ISOLATION, PURIFICATION AND RECOMBINANT METHOD OF PRODUCTION
- <130> MERCK 2332
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- <141> 2002-04-08
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Phe	Lys	Ala 195	Leu	His	Lys	Val	Leu 200	Glu	Lys	Tyr	Pro	Thr 205	Leu	Asn	Lys
Gly	Ser 210	Leu	Val	Gly	Pro	Asp 215	Val	Gly	Trp	Met	Gly 220	Val	Ser	Tyr	Val
Lys 225	Gly	Leu	Ala	Asp	Gly 230	Ala	Gly	Asp	Leu	Val 235	Thr	Ala	Phe	Thr	Leu 240
His	Gln	Tyr	Tyr	Phe 245	Asp	Gly	Asn	Thr	Ser 250	Asp	Val	Ser	Thr	Tyr 255	Leu
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Asn	Asn	Val	Lys	Val 325	Val	Ile	Arg	Gln	Thr 330	Ile	Tyr	Asn	Gly	Tyr 335	Туг
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Met	His	Val 355	His	Asn	Ser	Leu	Val 360	Gly	Asn	Thr	Val	Phe 365	Lys	Val	Asp
Val	Ser 370	Asp	Pro	Thr	Asn	Lys 375	Ala	Arg	Val	Tyr	Ala 380	Gln	Cys	Thr	Lys
Thr 385	Asn	Ser	Lys	His	Thr 390	Gln	Ser	Arg	Tyr	Tyr 395	Lys	Gly	Ser	Leu	Thr 400
Ile	Phe	Ala	Leu	Asn 405	Val	Gly	Asp	Glu	Asp 410	Val	Thr	Leu	Lys	Ile 415	Asp
Gln	Tyr	Gly	Gly 420	Lys	Lys	Ile	Tyr	Ser 425	Tyr	Ile	Leu	Thr	Pro 430	Glu	Gly
Gly	Gln	Leu 435	Thr	Ser	Gln	Lys	Val 440	Leu	Leu	Asn	Gly	Lys 445	Glu	Leu	Lys
Leu	Val	Ser	Asp	Gln	Leu	Pro	Glu	Leu	Asn	Ala	Asn	Glu	Ser	Lys	Thi

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Ala Asn Val Glu Ala Cys Lys Lys
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						aat Asn										528
						aat Asn										576
		_	_			gtt Val									_	624
	_		_			gat Asp 215									_	672
aag Lys 225	gga Gly	ttg Leu	gca Ala	gac Asp	gag Glu 230	gcr Ala	ggt Gly	gac Asp	cat His	gta Val 235	ack Thr	gct Ala	ttt Phe	aca Thr	ctc Leu 240	720
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_	_				_	aag Lys	_					_				816
_	_	_		_		cca Pro			_	_						864
~		_				aac Asn 295	_			_	-	_		_		912
	_					aca Thr		_	_	_						960
		_	_		_	ata Ile	_			_						1008
			_			act Thr			_		_	_				1056
		_				ttg Leu	_							_	-	1104

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tta Leu	gtg Val 450	tct Ser	gat Asp	cag Gln	tta Leu	cca Pro 455	gaa Glu	cta Leu	aat Asn	gca Ala	gat Asp 460	gaa Glu	tcc Ser	aaa Lys	aca Thr	1392
tct Ser 465	ttc Phe	acc Thr	tta Leu	tcc Ser	cca Pro 470	aag Lys	aca Thr	ttt Phe	ggt Gly	ttt Phe 475	ttt Phe	gtt Val	gtt Val	tcc Ser	gat Asp 480	1440
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Leu	Asn 130	Ala	Glu	Val	Arg	Thr 135	Gly	Tyr	Glu	Ile	Gly 140	Lys	Lys	Met	Thr
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Asp	His	Thr	Ser 180	Ala	His	Asn	Leu	Thr 185	Glu	Lys	Gln	Val	Gly 190	Glu	Asp
Phe	Lys	Ala 195	Leu	His	Lys	Val	Leú 200	Glu	Lys	Tyr	Pro	Thr 205	Leu	Asn	Lys
Gly	Ser 210	Leu	Val	Gly	Pro	Asp 215	Val	Gly	Trp	Met	Gly 220	Val	Ser	Xaa	Val
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t	tg eu	ctg Leu 50	Glu	gga Gly	ctt Leu	tct Ser	cct Pro	Gly	tac Tyr	tto Phe	agg Arg	gtt Val 60	. Gly	gga Gly	acg Thr	ttt Phe	192
Ą	jcc 1a 65	Asn	cgg Arg	ctg Leu	ttt Phe	ttt Phe 70	Asp	ttg Leu	gac Asp	gaa Glu	aat Asn 75	Asr	aag Lys	tgg Trp	aar Lys	gat Asp 80	240
t	at 'yr	tgg Trp	g gct Ala	ttt Phe	aaa Lys	Asp	aaa Lys	acc Thr	Pro	gaa Glu	Thr	gcg Ala	g aca Thr	ata : Ile	aca Thr	agg Arg	·288

aga Arg	tgg Trp	ctg Leu	ttc Phe 100	aga Arg	aaa Lys	caa Gln	aat Asn	aat Asn 105	ctg Leu	aaa Lys	aag Lys	gag Glu	act Thr 110	ttt Phe	gac Asp	33	36
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tca Ser	aaa Lys	ggt Gly	tac Tyr	gga Gly 165	gac Asp	aat Asn	atc Ile	gat Asp	tgg Trp 170	gaa Glu	ctt Leu	999 999	aat Asn	gga Gly 175	ccg Pro	5	28
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Ту1 305		Ser	Gly	/ Phe	Leu 310		Leu	. Asp	Lys	315		Leu	ser	Ala	Ala 320
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#### IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: MERCK 2332

In re patent application of

KORDOWICZ, MARIA et al.

Serial No. 10/009,500

Filed: April 8, 2002

FOT: HYALURONIDASE FROM THE HIRUDINARIA MANILLENSIS, ISOLATION, PURIFICATION AND RECOMBINANT METHOD OF PRODUCTION

## STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents Washington, D.C. 20231
Box SEQUENCE

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

- the submission, filed herewith in accordance with 37
   C.F.R. § 1.821(q), does not include new matter;
- 2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and
- all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

Serial No. 10/009,500

States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

James A. Coburn

Date

HARBOR CONSULTING

Intellectual Property Services 1500A Lafayette Road Suite 262 Portsmouth, N.H.

800-318-3021

#### SEQUENCE LISTING

- <110> KORDOWICZ, MARIA
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Gly	Gln	Leu 435	Thr	Ser	Gln	Lys	Val 440	Leu	Leu	Asn	Gly	Lys 445	Glu	Leu	Lys
Leu	Val 450	Ser	Asp	Gln	Leu	Pro 455	Glu	Leu	Asn	Ala	Asn 460	Glu	Ser	Lys	Thr

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														wac Tyr		672
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gat Asp	gcc Ala	aca Thr	tac Tyr 260	ttt Phe	aag Lys	aag Lys	ctg Leu	caa Gln 265	caa Gln	cta Leu	ttt Phe	gat Asp	aaa Lys 270	gtg Val	aaa Lys	816
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Tyr Trp Ala Phe Lys Asp Lys Thr Pro Glu Thr Ala Thr Ile Thr Arg

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Ser	Lys	Gly	Tyr	Gly 165	Asp	Asn	Ile	Asp	Trp 170	Glu	Leu	Gly	Asn	Glu 175	Pro
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Gly	Leu	Leu	Asp 340	Lys	Asn	Thr	Leu	Glu 345	Pro	Asn	Pro	Asp	Tyr 350	Trp	Leu
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Val	Ser 370	Asp	Pro	Thr	Asn	Lys 375	Ala	Arg	Val	Tyr	Ala 380	Gln	Cys	Thr	Lys
Thr	Asn	Ser	Lys	His	Thr		Ser	Arg	Tyr	Tyr	Lys	Gly	Ser	Leu	Thr

Ile Phe Ala Leu Asn Val Gly Asp Gly Asp Val Thr Leu Lys Ile Gly

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Leu	Val 450	Ser	Asp	Gln	Leu	Pro 455	Glu	Leu	Asn	Ala	Asp 460	Glu	Ser	Lys	Thr	
Ser 465	Phe	Thr	Leu	Ser	Pro 470	Lys	Thr	Phe	Gly	Phe 475	Phe	Val	Val	Ser	Asp 480	
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<220>

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Lys 225	Gly	Leu	Ala	Asp	Glu 230	Ala	Gly	Asp	His	Val 235	Thr	Ala	Phe	Thr	Leu 240

Lys Gly Leu Trp Ser Phe Val Asp Ile Thr Ser Pro Lys Leu Phe Lys

Asp Ala Thr Tyr Phe Lys Lys Leu Gln Gln Leu Phe Asp Lys Val Lys 260 265 270

His Gln Tyr Tyr Phe Asp Gly Asn Thr Ser Asp Val Ser Ile Tyr Leu

Asp Val Leu Lys Asp Ser Pro His Lys Asp Lys Pro Leu Trp Leu Gly 275 280 285

Glu Thr Ser Ser Gly Tyr Asn Ser Gly Thr Glu Asp Val Ser Asp Arg 290 295 300

Tyr Val Ser Gly Phe Leu Thr Leu Asp Lys Leu Gly Leu Ser Ala Ala 305 310 315 320

Asn Asn Val Lys Val Val Ile Arg Gln Thr Ile Tyr Ser Gly Tyr Tyr 325 330 335

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<sup>&</sup>lt;211> 488

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15

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#### ESIGNATED/ELECTED OFFICE (DO/EO/US) IN THE UNITED STE

International Application No.

PCT/EP00/05181

International Filing Date

6 JUNE 2000

U.S. Serial No.

10/009,500

Deposit Date U.S. Nat'l Phase

**10 DECEMBER 2001** 

Priority Date(s) Claimed

12 JUNE 1999

Applicant(s)

KORDOWICZ, Maria, et al.

Title: HYALURONIDASE FROM THE HIRUDINARIA MANILLENSIS, ISOLATION, PURIFICATION AND

RECOMBINANT METHOD OF PRODUCTION

# RESPONSE TO NOTIFICATION OF MISSING REQUIREMENTS **UNDER 35 U.S.C. § 371** IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Commissioner for Patents **Box PCT** Washington, D.C. 20231

Sir:

In response to the Notification of Missing Requirements mailed 7 FEBRUARY 2002, attached is a disk and paper copy of the sequence listing and the Notification of Missing Requirements.

Applicants confirm that the disk and paper copy of the sequence listing are identical.

The Patent and Trademark Office is authorized to deduct any additional fees from, or credit any overpayments to, counsel's deposit account No. 13-3402, a copy of this paper being attached.

Respectfully submitted

Anthony J. Zelano Reg. No.27, 969

Attorney for Applicants

MILLEN, WHITE, ZELANO & BRANIGAN, P.C.

Arlington Courthouse Plaza J

2200 Clarendon Boulevard, Suite 1400

Arlington, Virginia 22201

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703-243-6410 Internet Address:@mwzb.com

Filed: 8 APRIL 2002

## SEQUENCE LISTING

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Page 1

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96

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DUE DATE \_

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UNITED STATES PATENT AND TRAI	DEMARK OFFICE	United States Patient See Traderials Cities and States Patient See Traderials Cities (Cities Cities
	FIRST NAMED APPLICANT	ATTY, DOCKET NO.
U.S. APPLICATION NUMBER NO. 10/009,500	Maria Kordowicz	INTERNATIONAL APPLICATION NO.
		1.A FILING DATE PRIORITY DATE 06/06/2000 06/12/1999
23599 MILLEN, WHITE, ZELANO & BRANIGAN 2200 CLARENDON BLVD.		CONFIRMATION NO. 491
SUITE 1400 ARLINGTON, VA 22201 CASE ACTION	Missing Reg	371 FORMALITIES LETTER  *OC000000007425790*

# NOTIFICATION OF MISSING REQUIREMENTS UNDER 35 U.S.C. 371 IN THE UNITED Date Mailed: 02/07/2002 STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the IB to the United States Patent and Trademark Office as an Elected Office (37 CFR 1.495):

- U.S. Basic National Fees
- **Priority Document**
- Assignee Statement
- Biochemical Sequence Listing
- Copy of IPE Report
- Copy of references cited in ISR
- Copy of the International Application
- Copy of the International Search Report
- Oath or Declaration
- Preliminary Amendments

CASE -ACTION -DUE DATE -

The following items MUST be furnished within the period set forth below in order to complete the requirements f

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTH FROM THE DA acceptance under 35 U.S.C. 371: OF THIS NOTICE OR BY 22 or 32 MONTHS (where 37 CFR 1.495 applies) FROM THE PRIORITY DATE F( THE APPLICATION, WHICHEVER IS LATER. FAILURE TO PROPERLY RESPOND WILL RESULT IN

The time period set above may be extended by filing a petition and fee for extension of time under the provision of the pro ABANDONMENT. of 37 CFR 1.136(a).

The following items MUST be furnished within the period set forth below:

• The nucleotide and/or amino acid sequence disclosure contained in this application does not comply with requirements for such a disclosure as set forth in 37 CFR 1.821-1.825 for the following reason(s):

Dufd 2/11/02-12/16

- A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).
- APPLICANT MUST PROVIDE:
  - An initial or substitute computer readable form (CRF) of the "Sequence Listing."
  - A statement that the contents of the paper or compact disc and the computer readable form are the same and, where applicable, include no new matter, as required by 37 CFR 1.821(e), 1.821(f), 1.821(g), 1.825(b) or 1.825(d).
- For questions regarding compliance to 37 CFR 1.821-1.825 requirements, please contact:
  - For Rules Interpretation, call (703) 308-4216
  - To Purchase Patentin Software, call (703) 306-2600
  - For Patentin Software Program Help, call (703) 306-4119 or e-mail at patin21help@uspto.gov or patin3help@uspto.gov
  - A copy of the "Sequence Listing" in computer readable form has not been submitted as required by 37 CFR 1.821(e).

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

A copy of this notice MUST be returned with the response.

SHAKEEL AHMED

Telephone: (703) 305-3659

#### PART 1 - ATTORNEY/APPLICANT COPY

U.S. APPLICATION NUMBER NO.	INTERNATIONAL APPLICATION NO.	 · ATTY, DOCKET NO.
10/009,500	PCT/EP00/05181	MERCK 2332

FORM PCT/DO/EO/905 (371 Formalities Notice)

# IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

International Application No.

PCT/EP00/05181

International Filing Date

6 JUNE 2000

Priority Date(s) Claimed

12 JUNE 1999

Applicant(s) (DO/EO/US)

KORDOWICZ, Maria, et al.

Title: HYALURONIDASE FROM THE *HIRUDINARIA MANILLENISIS*, ISOLATION, PURIFICATION AND RECOMBINANT METHOD OF PRODUCTION

# PRELIMINARY AMENDMENT

Commissioner for Patents Washington, D.C. 20231

SIR:

Prior to calculating the national fee, and prior to examination in the National Phase of the above-identified International application, please amend as follows:

#### IN THE CLAIMS:

- 4. (Amended) A protein according to claim 1 having an isoelectric point of 7.2 8.0.
- 5. (Amended) A protein according to claim 1 having the amino acid sequence given in Fig. 7 and SEQ ID No. 1.
- 6. (Amended) A protein according to claim 1 having a specific enzymatic activity of > 100 kU/mg protein.

- 7. (Amended) A process for isolating and purifying the protein as defined in claim 1 comprising the following steps
  - (i) homogenization of heads of leeches of the species *Hirudinaria manillensis* with an acid buffer and centrifugation,
  - (ii) ammonium sulfate precipitation of the supernatant of step (i),
  - (iii) cation exchange chromatography,
  - (iv) concanavalin A affinity chromatography
  - (v) hydrophobic interaction chromatography
  - (vi) affinity chromatography on matrices coated with hyaluronic acid fragments
  - (vii) gel permeation chromatography, and optionally
  - (viii) enzymatic or chemical de-glycosylation of the purified protein.
- 10. (Amended) A DNA sequence coding for a protein of claim 1.
- 14. (Amended) An expression vector comprising a DNA sequence of claim 10.
- 15. (Amended) A host cell suitable for the expression of a protein of claim 12 which was transformed with a vector comprising a DNA sequence for a protein comprising any nucleotide sequence depicted in Fig. 8 (SEQ. ID No. 2), Fig. 9 (SEQ. ID No. 4) and Fig. 10 (SEQ ID No. 6).
- 16. (Amended) A protein according to any of claim 1 as a medicament.
- 20. (Amended) The use of a protein according to claim 1 in the manufacture of a medicament for treating myocardial, cardiovascular and thrombotic disorders and tumors.

## **REMARKS**

The purpose of this Preliminary Amendment is to eliminate multiple dependent claims in order to avoid the additional fee. Applicants reserve the right to reintroduce claims to canceled combined subject matter.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached pages are captioned "Version With Markings to Show Changes Made".

Respectfully submitted,

Anthony J. Zelano, Reg. No. 27,969

Attorney for Applicants

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Email: zelano@mwzb.com

AJZ:kmo

# **VERSION WITH MARKINGS TO SHOW CHANGES MADE**

Claims 4-7, 10, 14-16 and 20 have been amended as follows:

- 4. (Amended) A protein according to any of claims 1 3 having an isoelectric point of 7.2 8.0.
- 5. (Amended) A protein according to any of claims 1—4 having the amino acid sequence given in Fig. 7 and SEQ ID No. 1.
- 6. (Amended) A protein according to claims 1=5 having a specific enzymatic activity of > 100 kU / mg protein.
- 7. (Amended) A process for isolating and purifying the protein as defined in claims 1—6 comprising the following steps
- (i) homogenization of heads of leeches of the species *Hirudinaria manillensis* with an acid buffer and-centrifugation,
  - (ii) ammonium sulfate precipitation of the supernatant of step (i),
  - (iii) cation exchange chromatography,
  - (iv) concanavalin A affinity chromatography
  - (v) hydrophobic interaction chromatography
  - (vi) affinity chromatography on matrices coated with hyaluronic acid fragments
  - (vii) gel permeation chromatography, and optionally
  - (viii) enzymatic or chemical de-glycosylation of the purified protein.
- 10. (Amended) A DNA sequence coding for a protein of claim 1 and 9.
- 14. (Amended) An expression vector comprising a DNA sequence of claim 10 or 11.
- 15. (Amended) A host cell suitable for the expression of a protein of claim 12 or 13 which was transformed with a vector of claim 14. vector comprising a DNA sequence for a protein comprising any nucleotide sequence depicted in Fig. 8 (SEQ. ID No. 2), Fig. 9 (SEQ. ID No. 4) and Fig. 10 (SEQ. ID No. 6).

- 16. (Amended) A protein according to any of claims 1 -6,8,9,12 and 13 as a medicament.
- 20. (Amended) The use of a protein according to any of claims 1 6,8,9,12 and 13 in the manufacture of a medicament for treating myocardial, cardiovascular and thrombotic disorders and tumors.

WO 00/77221

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# Hyaluronidase from the *Hirudinaria manillensis*, isolation, purification and recombinant method of production

The present invention relates to the isolation, purification and characterization of a novel hyaluronidase which derives from the tropical leech *Hirudinaria manillensis*. Therefore, according to this invention the new enzyme is called "manillase". The invention is furthermore concerned with the recombinant method of production of manillase which includes the disclosure of DNA and amino acid sequences as well as of expression vectors and host systems. Finally, the invention relates to the use of manillase for therapeutic purposes, for example, for the treatment of myocardial diseases, thrombotic events and tumors.

Hyaluronic acid or hyaluronan (HA) is a linear unbranched high molecular-weight (2-6 x 10<sup>6</sup>) glycosaminoglycan, composed of a repeating disaccharide structure GlcNAc(ß1-4)GlcUA. Its carboxyl groups are fully ionized in the prevailing pH of extracellular fluids, whether normal or pathological. HA belongs together with the chondroitin sulphates, keratan sulfates and heparins to the group of glycosaminoglycans (Jeanloz R. W., *Arthr Rheum.*, 1960, 3, 233-237). In contrast with other unmodified glycosaminoglycans (GAG), it has no sulfate substitution or covalently linked peptide, and its chain length and molecular weight are usually very much greater. HA is ubiquitously distributed in connective tissues and has been found in virtually all parts of the body after introduction of improved fixation method (Hellström S. et al., 1990, *Histochem. J.*, 22, 677-682) and the specific histochemical method with the use of hyaluronan-binding peptides (HABP). It is present during development and maturity in tissues of neuroectodermal origin as well.

The term hyaluronidase refers generally and according to this invention to an enzyme, which acts on hyaluronic acid, irrespective of activity towards other substrates.

Hyaluronidase was first isolated from microorganisms and later from mammalian testis which is now its main source (Meyer K. in *The Enzyme*, 1971, 307).

According to the reaction mechanism, hyaluronidases were divided into three main groups.

In the first group microbial enzymes are combined that act on their substrates by  $\beta$ -elimination producing  $\Delta$ -4,5-unsaturated disaccharides. The enzyme must therefore be named hyaluronate lyases, EC 4.2.99.1.

The second group, hyaluronoglucosaminidase or testicular-type hyaluronidase (EC 3.2.1.35) acts as an endo-N-acetyl-ß-D-hexosaminidase degrading HA to smaller fragments, in the first place tetrasaccharide with the hexosamine moiety at the free reducing end. Enzymes with similar properties to the testis hyaluronidase have been obtained from tadpoles, snake venom, bee venom, numerous animal tissues, human serum and other sources. It is well know that hyaluronidase from testis has also transglycosylase activity (Weissman B. et al., *J. Biol. Chem.*, 1954, 208, 417-429). The enzymes belonging to this group of hyaluronidases exhibit enzymatic activity not only towards hyaluronate but also towards chondroitin-4-sulfate, chondroitin-6-sulfate, chondroitin and dermatan sulfate.

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The third group consists of hyaluronoglucuronidase (EC 3.2.1.36), which acts as an endo-ß-glucuronidase. This enzyme was isolated from the Hirudo medicinalis 20 leeches (Yuki H. & Fishman W.H.; J. Biol. Chem. 1963, 238, 1877-79) and is absolutely specific for HA. Chondroitin sulfate, dermatan and heparin are not substrates for this hyaluronidase. It degrades only hyaluronic acid to tetrasaccharide with the glucuronic acid at the free reducing end (Linker A. et al., J. Biol. Chem., 1960, 235, 924-27). Opposite to mamalian endo-ß-25 glucosaminidases, heparin has no influence on the activity of this leech hyaluronidase. Therefore, it can be coadministered to a patient together with a heparin and its derivatives extensively used as anticoagulants. A hyaluronic acid specific endo-beta-glucuronidase (called "Orgelase") from species (Poecilobdella granulosa) of the sub-family Hirudinariinae (including the genera Hirudinaria, Illebdella, Poecilodbella, Sanguisoga) of buffalo leeches was disclosed in EP 0193 330 having a molecular weight of about 28,5.

Hyaluronidases have many practical in vivo and in vitro applications. Intravenous administration of hyaluronidase has been proposed for treatment of myocardial infraction (Kloner R.A et al., Circulation, 1978, 58, 220-226; Wolf R.A. et al., Am. J. Cardiol., 1984, 53, 941-944; Taira A. et al., Angiology, 1990, 41, 1029-1036). Myocardial infraction represents a common form of non-mechanical injury: namely severe cell damage and death, caused in this instance by sudden cellular hypoxia. In an experimental myocardial infraction induced in rats (Waldenström A. et al., 1991, J. Clin. Invest., 88, 1622-1628), HA content of the injured (infracted area) heart muscle increased within 24 h to reach nearly three times normal after 3 days, and was accompanied by interstitial oedema. The relative water content of infracted areas also increased progressively reaching a maximum value by day 3 and was strongly correlated with the HA accumulation. The same association of increased HA content with oedema has been observed in experimental heart and renal transplant rejection (Hällgren R. et al., J. Clin. 15 Invest., 1990, 85, 668-673; Hällgren R. et al., J. Exp. Med., 1990, 171, 2063-2076) in rejection of human renal transplants (Wells A. et al. Transplantation, 1990, 50, 240-243), lung diseases (Bjermer A. et al., Brit. Med. J., 1987, 295, 801-806) and in idiopathic interstitial fibrosis (Bjermer A. et al., Thorax, 1989, 44, 126-131). All these studies provide not only evidence of increased HA in acute inflammation, but demonstrate its part in the local retention of fluid mainly responsible for the tissue swelling and influencing both the mechanical and electrophysiological functions of heart.

These results can explain the mechanism of the action of hyaluronidases used in clinical trials. It was reported that hyaluronidase treatment limited cellular damage during myocardial ischemia in rats, dogs and man (Maclean D. et al. *Science*, 1976, 194, 199). The degradation of the HA can be followed by the reduction of tissue water accumulation, reduction of the tissue pressure and finally better perfusion.

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It has been shown that hyaluronidases as well as hyaluronidase containing extracts from leeches can be used for other therapeutic purposes. Thus, hyase therapy, alone or combined with cyclosporine, resulted in prolonged graft survival (Johnsson C. et al. *Transplant Inter*. in press). Hyases ("spreading factor") in the

broadest sense are used to increase the permeability of tissues for enhancing the diffusion of other pharmacological agents (e.g. in combination with cytostatics in the treatment of cancer tumors). Furthermore, it could be demonstrated that hyaluronidases are useful in tumor therapy acting as angiogenesis inhibitor and as an aid to local drug delivery in the treatment of tumors, for the treatment of glaucoma and other eye disorders and as adjunct to other therapeutic agents such as local anaesthetics and antibiotics. A general overview of the therapeutic use and relevance is given in the review article of Farr et al. (1997, Wiener Medizinische Wochenschrift, 15, p. 347) and literature cited therein.

Therefore, there is a need for an active compound such as hyaluronidase. However, the known and available hyaluronidases are either not stable (hyaluronidase from Hirudo medicinalis, Linker et. al., 1960, J. Biol. Chem. 235, p. 924; Yuki and Fishman, 1963, J. Biol. Chem. 238, p. 1877) or they show a rather low specific activity (EP 0193 330, Budds et al., 1987, Comp. Biochem.

Physiol., 87B, 3, p. 497). Moreover, none of the known hyaluronidases are available in recombinant form which is an essential prerequisite for intensive commercial use.

This invention discloses now for the first time a new hyaluronidase which was isolated and purified from Hirudonaria mannilensis as well as a recombinant version of said enzyme obtained by bioengineering techniques.

Thus, it is an object of this invention to provide a purified protein isolated from the leech species *Hirudinaria manillensis* having the biological activity of a hyaluronidase which is not influenced in its acvtivity by heparin and characterized in that it has a molecular weight of 53 – 60 kD dependent on glycosylation. The new protein, which is called "manillase", is glycosylated in its native form having a molecular weight of ca. 58 kD (±2kD) and four glycoforms. However, the non-glycosylated protein is object of the invention as well, obtainable by enzymatic or chemical cleavage of the sugar residues according to standard techniques. The non-glycosylated enzyme of the invention has a molecular weight of about 54 (±2) as measured by SDS-PAGE.

Direct comparison shows that the hyaluronidase disclosed in EP 0193 330 ("orgelase") has under the same conditions a molecular weight of about 28 and contains a lot of impurities such as hemoglobin.

Native manillase according to this invention has a pH optimum of 6.0 - 7.0, an isoelectric point of 7.2 - 8.0 and has the amino acid sequence depicted in Fig. 7.

Surprisingly manillase obtained by a preparative purification procedure (see below) has an extremely high specific activity of 100 – 150, preferably of 110 – 140 (WHO) kU/mg protein whereas the specific activity of orgelase is about 1,2 kU/ mg only. Moreover, orgelase has a lower pH optimum (5.2 - 6.0) as compared with manillase. Manillase is not influenced, like orgelase, by heparin.

Furthermore it is an object of the invention to provide a process for isolating and purifying manillase comprising the following steps

- 15 (i) homogenization of heads of leeches of the species *Hirudinaria manillensis* with an acid buffer and centrifugation,
  - (ii) ammonium sulfate precipitation of the supernatant of step (i),
  - (iii) cation exchange chromatography,
  - (iv) concanavalin A affinity chromatography
- 20 (v) hydrophobic interaction chromatography
  - (vi) affinity chromatography on matrices coated with hyaluronic acid fragments
  - (vii) gel permeation chromatography, and optionally
  - (viii) enzymatic or chemical deglycosylation of the purified protein.
- The process steps disclosed above guarantee that the protein according to the invention can be obtained with such a high biological enzyme activity. Therefore, it is a further object of this invention to provide a protein having the biological activity of a hyaluronidase which is not influenced in its activity by heparin and having a molecular weight of 53 60 dependent on glycosylation which is obtainable by the process steps indicated above and in the claims and which has preferably a specific enzyme activity of > 100 kU/ mg protein. The term "unit" relates below and above to "international units" (IU).

The invention discloses a process of making recombinant manillase which includes respective DNA molecules, vectors and transformed host cells. Therefore, it is an object of this invention to provide a DNA sequence coding for a protein having the properties of native manillase.

It could be also shown, that at least three further clones with slightly different DNA sequences could be selected which are coding for proteins with manillase (hyaluronidase) properties having slightly different amino acid sequences.

The specified clones have the DNA sequences depicted in Fig. 8, 9 and 10 (upper sequence) which are an object of this invention too as well as expression vectors containing said sequences and host cells which were transformed with said vectors.

In addition, it is object of this invention to provide a recombinant protein with the biological activity of a hyaluronidase and a molecular weight of 55 – 59 kD dependent on glycosylation having any amino acid sequence depicted in Fig. 8, 9 and 10 (lower sequence) or a sequence which has a homology to said sequences of at least 80%. The term "manillase" includes all these proteins having the above-specified properties.

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The native as well as the recombinant protein(s) may be used as a medicament which can be applied to patients directly or within pharmaceutical compositions. Thus, it is a further aspect of this invention to provide a recombinant or native protein as defined above and below applicable as a medicament and a respective pharmaceutical composition comprising said protein and a pharmaceutically acceptable diluent, carrier or excipient therefor.

The pharmaceutical compositions of the invention may contain additionally further active pharmaceutical compounds of a high diversity. Preferred agents are anticoagulants which do not inhibit or influence the biological and pharmacological activity of the protein according to the invention. Such anticoagulants can be, for example, heparin, hirudin or dicoumarin, preferably, heparin. Thus, it is an object of the present invention to provide a pharmaceutical

composition comprising additionally a pharmacologically active compound, preferably heparin.

In connection with use in human or veterinary therapy the protein according to
this invention acts preferably as dispersal agent ("spreading" factor) or supports
penetration through tissue and skin. Thus, manillase can be used as an adjunct
of other substances (such as an local anaesthetic) e.g. in the field of
chemotherapy of tumors, for treatment of disorders and diseases with respect to
acute myocardial ischemia or infarction, for treatment of glaucoma and other eye
disorders, e.g. to improve the circulation of physiological fluids in the eye, for
treatment of skin and tissue grafts to remove congestion and improve circulation,
as drug delivery system through the skin, membranes, other tissue, as an agent
to remove the hyaluronic acid capsule surrounding certain pathogenic
microorganisms or certain tumors and cancerous tissues, and as an inhibitor of
angiogenesis which can be used as anti-thrombotic and anti-tumor agent.

Therefore, the use of manillase as defined above and below in the manufacture of a medicament for treating especially myocardial, cardiovascular and thrombotic disorders and tumors is an object of this invention.

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As used herein, the term "pharmaceutically acceptable carrier" means an inert, non toxic solid or liquid filler, diluent or encapsulating material, not reacting adversely with the active compound or with the patient. Suitable, preferably liquid carriers are well known in the art such as sterile water, saline, aqueous dextrose, sugar solutions, ethanol, glycols and oils, including those of petroleum, animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil and mineral oil.

The formulations according to the invention may be administered as unit doses containing conventional non-toxic pharmaceutically acceptable carriers, diluents, adjuvants and vehicles which are typical for parenteral administration.

The term "parenteral" includes herein subcutaneous, intravenous, intra-articular and intratracheal injection and infusion techniques. Also other administrations such as oral administration and topical application are suitable. Parenteral

compositions and combinations are most preferably adminstered intravenously either in a bolus form or as a constant fusion according to known procedures. Tablets and capsules for oral administration contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, and wetting agents. The tablets may be coated according to methods well known in the art.

Oral liquid preparations may be in the form of aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or another suitable vehicle before use. Such liquid preparations may contain conventional additives like suspending agents, emulsifying agents, non-aqueous vehicles and preservatives.

Topical applications may be in the form of aqueous or oily suspensions, solutions, emulsions, jellies or preferably emulsion ointments.

Unit doses according to the invention may contain daily required amounts of the protein according to the invention, or sub-multiples thereof to make up the desired dose. The optimum therapeutically acceptable dosage and dose rate for a given patient (mammals, including humans) depends on a variety of factors, such as the activity of the specific active material employed, the age, body weight, general health, sex, diet, time and route of administration, rate of clearance, enzyme activity (units/mg protein), the object of the treatment, i. e., therapy or prophylaxis and the nature of the disease to be treated.

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Therefore, in compositions and combinations such as with anticoagulants like heparin in a treated patient (in vivo) a pharmaceutical effective daily dose of the protein of this invention (manillase) is between about 0.01 and 100 mg/kg body weight (based on a specific activity of 100 kU/mg), preferably between 0.1 and 10 mg/kg body weight. According to the application form one single dose may contain between 0.5 and 10 mg of manillase.

The concentration of e.g. heparin when administered together with manillase is typically 500 - 4000 U (IU) over one day, however, may be increased or diminished if necessary.

The purification of manillase of the invention was achieved as described in detail in the examples. Table 1 depicts a preparative purification scheme of manillase. Table 2 shows the process of enrichment of the protein according to the invention and Table 3 indicates the comparison of manillase with known leech hyaluronidases.

An enzyme, named manillase, cleaving hayaluronic acid has been isolated from the heads of Hirudinaria manillensis leeches and purified to homogeneity. This hyaluronidase was purified using acid-extraction, ammoniumsulfate precipitation, followed by successive chromatography on cation exchanger, Concanavalin A-Sepharose, Propyl-Fractogel, Hyaluronan fragments-Sepharose and Diol-LiChrospher columns. The hyaluronan fragments were prepared by the cleavage of the native hyaluronan with the aid of bovine testes hyaluronidase. After purification and characterization of the fragments, the affinity matrices were prepared as indicated below. Such affinity matrices were applied for the first time for purification of the hyaluronidase. This high-performance chromatography is a technique for fast and efficient purification of hyaluronan binding proteins. The recovery of enzyme activity after each step of purification was reasonably high. The results of the three independent preparative purifications were comparable. They resulted in highly active samples possessing between 20 to 160 kU/mg dependent on the degree of purification. In comparison experiments known hyaluronidases were isolated as indicated in the prior art and their properties were compared with the protein according to this invention (Tab. 3).

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The hyaluronidase purified according to the scheme of Tab. 1 differs from other leech hyaluronidases described by other authors. A similar molecular weight was obtained under non-dissociating conditions (any ß mercaptoethanol), indicating that manillase is a single subunit enzyme in common with a wide range of hyaluronidase preparations from mammalian sources. This final preparation is a single subunit enzyme (Fig. 1) of apparent molecular weight 58 ± 2 determined with the aid of MALDI, with isoelectric point of 7,2 to 8,0.

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#### Preparative purification of manillase Tab. 1:

Preparation of the starting material Leeches from - Bangladesh - ~ 15 kg 1 Separation of the living animals Freezing of these animals Preparation of the heads

~ 1 kg leech heads Homogenization and Extraction\* Acid precipitation centrifugation\*\* Stage I - sample 36 % ammonium sulfate precipitation of supernatants centrifugation, dialysis\*\*

Stage II - sample

Cation exchange EMD (SO<sub>3</sub>-)\* Chromatography Dialysis\*\*\* Con A -affinity chromatography Dialysis\*\*\*\* Ţ Propyl - Fractogei chromatography\* Dialysis\*\*\*\* Hyaluronic acid fragments (HA) - affinity

chromatography Dialysis\*\*\*\* I

140 000 WHO Units Diol-LiChrospher chromatography\*\*\*\*

Reverse-Phase chromatography Analytic\*\*\*\*

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<u>Tab.2:</u> Purification of manillase (enrichement) from 1 kg of leech heads

Step of purification	Total protein Mg	Total activity kU	% recovery	Specific activity U/mg	Purification (fold)
Stage I supernatant after extraction and acid precipitation	31 700	633.3	100	20	1
Stage II supernatant after 36% ammonium sulfate precipitation	9 530	443.3	70	45	2.25
Cation exchange chromatography	426.7	332.5	52.5	770	38.5
Con A affinity - chromatography	41.0	166.2	26.2	4.000	200
Propyl-Fractogel chromatography	11.9	133.0	21.0	11 000	550
Hyaluronic acid fragments-Sepharose affinity chromatography	1.9	66.4	10.5	35 000	1 750
Diol-LiChrospher	0.307	33.2	5.2	108 000	5 400

<u>Tab.3:</u> Comparison of manillase with known leech hyaluronidases

	" <u>Manillase</u> "	Hvaluronidase	<u>Hyaluronidase</u>	"Orgelase"	
	Hirudinaria manillens.	H. medicinalis	H. medicinalis	P. granulosa	
	Invention	comparison	Linker et al.;	EP 0 193 330	
		experiment	(J.Biol.Chem, 1960)	Budds et al.	
specific activity					
WHO (IU)	140 000	~20 000	≤100	≤100	
units/mg		semipurified			
homogeneity	1 protein			mixture of	
SDS-PAGE	homogenous	Mixture of	no results available	many proteins	
MALDI	4 glycoforms	proteins		main impurity:	
				hemoglobin	
molecular	58,3 kD ± 2 kD	n. d.	not reported	28,5 ± 3 kD	
weight				1	
amino acid	determined	n. d.	not reported	not determined	
sequence					
pH optimum	6.0 - 7.0	6.0 - 7.0	not reported	5,2 - 6.0	
pI	7.5 - 8,0	n. d.	n. d.	n. d.	
	binding to Propyl-	no binding to			
hydrophobicity	HIC at 2 M	Propyl-HIC at			
	ammonium sulfate	2 M ammonium			
		sulfate			
activity					
reduction by	no influence	not determined	no influence	no influence	
heparin	·				
Stability					
	stable	Unstable			
at +4°C	after 7 days	100% loss of			
	~ 75% activity retained	activity after 7			
4		days incubation			
	stable	Unstable		relatively stable	
at +37°C	after 7 days	100% loss of			
	~ 60% activity retained	activity after 7			
		days incubation			
stability	stable	Unstable			
at +37°C in the	after 7 days	100% loss of	not reported	not tested	
presence of the		activity after 1			
dog's serum	retained	day incubation			
dog 5 serum					

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The asterisks in the tables mean information on activity determination and biochemical characterization (\* - \*\*\*\*\*).

The methods of activity determination and biochemical characterization used depend of the concentration of manillase in the analyzed samples. Therefore, they were successively extended by the appropriate techniques in the successive steps of purification.

- Activity determination turbidity reduction test
- \*\* Activity determination -turbidity reduction test
  - Protein content determination (E280, Pierce BCA method)
  - SDS PAGE (SDS Polyacrylamide Gel Electrophoresis)
  - Hemoglobin determination
- Activity determination -turbidity reduction test
  - Protein content determination (E280, Pierce BCA method)
  - SDS PAGE Western Blot (anti human hemoglobin antibody)
- 15 \*\*\*\* Activity determination -turbidity reduction test
  - Protein content determination (E280, Pierce BCA method)
  - SDS PAGE Western Blot anti human hemoglobin antibody,
  - SDS PAGE Western Blot anti Con A antibody
  - SDS PAGE Western Blot anti peptide antibodies
- 20 \*\*\*\*\* MALDI
  - Protein content determination (Pierce BCA method)
  - SDS PAGE Western Blot anti peptide antibodies

Binding of manillase to Concanavalin A shows that this hyaluronidase is a glycoprotein, whose sugar components are terminated with α-D-mannopyranosyl or α-D-glucopyranosyl and sterically related residues. Manillase-active samples showed two bands with almost identical RF values in SDS-PAGE. Longer SDS-PAGE and different running conditions were used for better separation of the bands. In these experiments two additional, weaker bands could be detected (Fig. 2). The N-terminal part all of them (30 amino acids) was individually sequenced and showed again no difference in the N-terminus. Following deglycosylation with the endo-F-glycosidase (PNGase) it was observed that all four bands resulted in a single band, with a reduction in MW of about 3.

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Therefore, it is quite likely that the observed differences in electrophoretic mobility are due to differences in the glycosylation pattern of manillase molecules. The neuraminidase, O-endo-glycosidase and neuraminidase plus O-glycosidase treatments have no influence on the molecular weight of the purified enzyme (Fig.

3). These results have shown that manillase contains at least one N-linked oligosaccharide chain. The O-linked carbohydrate chains could not be detected with the method used.

As the concluding purification step, the RP-chromatography was carried out.

Although the enzymatic activity could not be detected any more, the salts and peptide protease inhibitors could be removed (Fig. 4). The fractions containing protein were characterized further with the help of MALDI. The molecular weight of manillase determined with the aid of MALDI was 58,3.

- Heparin has no influence on the activity of this hyaluronidase (Fig. 5). Manillase is many fold more stabile than Hirudo medicinalis hyaluronidase (Fig. 6). Moreover, the samples of partly purified manillase showed very high stability in the dogs and rats plasma within the -20 to + 37 range.
- The preparation of HA-affinity matrices has been described in the literature (Tengblad A., *Biochim. Biophys. Acta*, 1979, **578**, 281-289). This HA-matrix was used for the purification of the cartilage hyaluronate binding proteins or proteoglycan protein-keratan sulfate core (Christner J. E., *Anal. Biochem.*, 1978, **90**, 22-32) from the same source. The HA-binding protein (HABP), purified with the aid of this affinity matrix, was used further in histochemical studies concerning the distribution of the hyaluronate receptors (Green S.J. et al., *J. Cell Science*, 1988, **89**, 145-156; Chan F. L. et al., *J. Cell. Biol.*, 1997, **107**, 289-301) or hyaluronan (Waldenström A. et al., 1991, *J. Clin. Invest.*, **88**, 1622-1628; Waldenström A. et al., *Eur. J. Clin. Invest.*, 1993, **23**, 277-282) in the tissues.

However, the method of the preparation of this gel developed in our laboratory enables one to produce gels of exactly defined concentration of HA-fragments (1 to 15 mg/ml). This, in turn, enables one to use such gels not only for purification of hyaluronan-binding proteins but also for their separation, by taking advantage

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of their different affinity to hyaluronan. This selective separation can be controlled by using of HA-fragments of different length. Such separation will enable one to better characterization many receptors of biological relevance (e. g. in oncology)

- 5 HA-matrices prepared according to the method described can be applied for the:
  - 1) purification of known HA-binding proteins
  - 2) purification of unknown HA-binding proteins
  - 3) identification of the new HA-binding proteins
  - 4) purification of hyaluronidases

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HA-fragments obtained by the method described in the present invention can be characterized with the use of modern analytical methods (NMR, MALDI-MS) and applied in the research on protein-protein interactions. Furthermore, these fragments can be used in the research concerning angiogenesis and neovascularization processes

# Short description of the figures:

- Fig. 1: SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE CBB staining) of the protein standard, manillase sample (after Diol-LiChrospher chromatography).
  - 1 wide range protein standard
  - 2 Manillase, 4µg
  - 3 Orgelase, 6 μg
  - 4 Hemoglobin, 40 μg
- 25 Fig. 2: a) SDS-PAGE (CBB staining) and
  - b) SDS-PAGE Western blot of four manillase-active samples (lines 3-6) after HA affinity chromatography. Rabbit P3-2A polyclonal anti-peptide antibody was used in this experiment.
  - Fig. 3: SDS-PAGE (CBB) of the following samples:
    - 1- LW-MM low weight molecular marker (BioRad)
    - 2- Manillase
    - 3- N-Glycosidase F (PNGase F)
    - 4- Manillase after treatment with PNGase F
    - 5- Manillase after treatment with O-glycosidase

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- 6- Manillase after treatment with O-glycosidase and neuraminidase
- 7- O-glycosidase and neuraminidase
- 8- molecular weight marker (MWM-prestained BioRad)
- Fig. 4: Reverse-Phase-Chromatography of
  - a) Ribonuclease standard
  - b) manillase sample (specific activity 140 kU/mg)
- Fig. 5: Influence of heparin on hyaluronidase activity of manillase ( ○ -) and bovine testes hyaluronidase (- - )

X-axis: IU heparin; Y-axis: % activity left

- Fig. 6: Stability measurement of hyaluronidases in buffer and plasma:
  - (a) manillase (4°C), (b) manillase (-20°C)
  - (c) manillase (37°C),
  - (d) bovine testes hyaluronidase (Y) and Hirudo medicinalis hyaluronidase (A)

X-axis: days of incubation; Y-axis: WHO (IU) units

- Fig. 7: Amino acid sequence of native manillase obtained by sequencing of the isolated and purified protein from Hirudinaria manillensis accordning the invention (corresponds to SEQ ID No. 1)
- Fig. 8: Nucleotide (upper lines) and amino acid sequence of a recombinant manillase clone (clone 21); (corresponds to SEQ ID. Nos. 2, 3)
- Fig. 9: Nucleotide (upper lines) and amino acid sequence of a recombinant manillase clone (clone 31); (corresponds to SEQ ID. Nos. 4, 5)
- Fig. 10: Nucleotide (upper lines) and amino acid sequence of a recombinant manillase clone (clone 31); (corresponds to SEQ ID. Nos. 6, 7)
- 25 Fig. 11: E. coli expression vector for manillase
  - Fig. 12: Baculo donor plasmid for manillase
  - Fig. 13: Yeast expression vector for manillase

The invention is described in detail by the following examples. However, these examples do not limit the invention to the general materials, methods, physical parameters, compounds, biological materials, expression vectors and hosts etc. used in the experiments and indicated in the examples. If not otherwise mentioned standard techniques well known in the prior art and generally available material were used.

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#### Example 1 (General Remarks):

A number of preliminary experiments were carried out using crude extracts of Hirudinaria manillensis in order to establish the purification procedure.

The following methods were chosen and verified: ammonium sulfate precipitation procedure, cation and anion exchange chromatography, affinity chromatography with the aid of Heparin-Fractogel, Con A-Sepharose, Hydrophobic Interaction Chromatography (HIC) on Octyl-Sepharose, Propyl- Phenyl-, Butyl-Fractogel, preparative isoelectric focusing and preparative electrophoresis.

The results show that acid and ammonium precipitation, cation exchange, Con A-Sepharose, Propyl-Fractogel HIC and Diol-LiChrospher and Hyaluronic acid fragments-Sepharose (HA-Sepharose) chromatography are suitable for the purification of the manillase. The HA-Sepharose matrix prepared in our laboratory was successfully used for the purification of this glycosidase.

All preparations were carried out in the cold unless otherwise mentioned.

The purification was done according to the scheme shown above (Tab. 1).

Example 2: - Preparation of the Starting Material for the Purification; Preparation of Leech Heads.

Hirudinaria manillensis leeches collected in Bangladesh were immediately shockfrozen and then stored at -40° to -80°. They were decapitated in frozen state, the weight of the heads amounting to ca. 5% of the body.

Example 3: - Extraction Procedure of Manillase from Leech Heads
In a representative purification, 1 kg of frozen leech heads were homogenized in a Waring Blender with 2500 ml of cold 0.1 M acetic acid buffer pH 4.0 containing 0,025% thimerosal and 17 mg/ml of trehalose (Merck KGaA, Art. No. 1.08216).

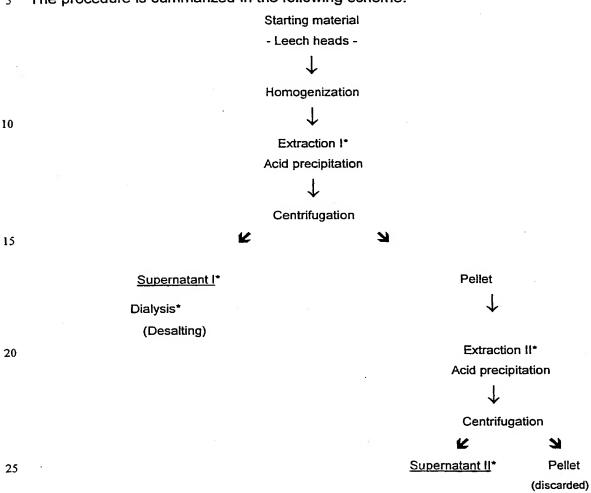
The homogenate was stirred gently and the following protease inhibitors were added immediately:

1. PMSF	1.7 mg/ml	10.0 mM
2.Leupeptin	10.0 μg/ml	20.0 μΜ
3.Pepstatin A	0.7 μg/ml	1 μΜ
4. EGTA	380.35 μg/m	11.0 mM
5.p-APMSF	40.0 μg/ml	20.0 μΜ

Stirring was continued for 4 hour in the cold and centrifuged at 4900 rpm for 20 minutes. The supernatant solution (supernatant I) was collected and pooled with supernatant II subsequently obtained by extracting the tissues pellet.

The pooled supernatants represent Stage I material.

The procedure is summarized in the following scheme:



\*Activity determination and biochemical characterization of the samples was performed with the aid of activity determination -turbidity reduction test and protein content determination (E<sub>280</sub>, Pierce BCA method, SDS - PAGE). It was impossible to measure the enzyme activity in the leech homogenate, because of the very high content of hemoglobins (measured with the hemoglobin determination kit, Merck KGaA, 13851) and other proteins. Moreover, the hyaluronidase activity could not be measured in the stage prior to the acid precipitation. The final specific activities (activity per mg of protein) of these

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extracts were about 10-30 WHO Units. According to SDS-PAGE, the crude extracts contained large amounts of different proteins, the major ones having a molecular weight of ~120, 55 -60, 45, 31, 28, 22, 15 and 14-10.

5 Example 4: - Ammonium Sulfate Precipitation Procedure of the Stage I Material Next, the ammonium sulfate precipitation procedure was chosen as the first step of the purification of manillase and resulted in a ~5-fold of enrichment of this enzyme.

Enzymatically inert material was precipitated from Stage I crude extract by adding slowly solid ammonium sulfate (Merck KGaA) to 36% w/v at +4°C. This mixture was stirred for 1 hour and centrifuged. The precipitate was discarded. The supernatant was dialyzed against running de-ionized water overnight, and 24 hours against 20 mM phosphate buffer pH 6.0. The final specific activities of these extracts were about 40 ~ 150 WHO Units. According to SDS-PAGE, the stage II extracts contain large amounts of different proteins.

# Example 5: - Cation Exchange Chromatography

The cation exchanger was used in a batch adsorption mode. An enzyme-rich dialyzed sample (stage II) was incubated overnight with 1 I Fractogel EMD SO<sub>3</sub><sup>-</sup> 650 (S) cation exchanger, Merck KGaA, Art. No. 16882. After the incubation was finished by centrifugation, the cation exchanger was washed with the buffer, centrifugated again and HPLC-Superformace column was filled with the gel. After washing the column with 20 mM phosphate buffer pH 4.9 the bound proteins were eluted from the column with the same sodium phosphate buffer pH 6.0 containing a linear 0 to 1 M gradient of NaCl. Fractions were collected every 3 min (9 ml) and the absorbance at 280 nm was monitored. Manillase was eluted at 0,15 to 0,18 M NaCl concentrations. The activities and protein contents of all fractions were measured and the fractions were pooled and dialyzed overnight against 20 mM phosphate buffer pH 6,0 containing sodium azide and 17 mg/ml trehalose.

Determination of the concentration of proteins, specific activities of the "pools", and SDS-PAGE analysis were carried out. In spite of very good yields (activity) and high specific activity (WHO activity units per mg of protein, corresponds to IU), a mixture of many proteins was still shown by the results of SDS-PAGE

analysis of the samples. The cation exchange chromatography with the aid of Fractogel EMD SO<sub>3</sub><sup>-</sup>650 (S) ® (Merck KGaA, Germany) resulted in a very high purification factor of ~ 10 to 50. This step is very effective in reducing hemoglobin impurities. Moreover, we have found that the batch procedure was a very useful initial step for handling large volumes of stage II supernatant (5 - 16 I).

Example 6: - Concanavalin A -Sepharose Affinity Chromatography The further purification of the enzyme-rich pools after cation exchanger was done with the aid of Con A lectin affinity chromatography. Commercially available Con A-Sepharose® from Pharmacia Biotech, Art. 17-0440-01, was washed with an acetic buffer 0.1 M + 0.5 M NaCl pH 8.0; 0.1 M boric acid + 0.1 % Triton X 100 pH 6.0 and finally with 0.1 M acetic buffer + 0.5 M NaCl pH 6.0. The sample was dialyzed overnight against 20 mM acetic buffer + 0.5 mM NaCl + 1 mM CaCl<sub>2</sub> +1 mM MgCL<sub>2</sub> pH 6.0 + 1 mM MnCl<sub>2</sub>, applied at room temperature to a 1000 ml Con A column and eluted 2 h with the 510 ml of 100 mM acetic acid buffer + 0.5 M NaCl + 1 mM CaCl<sub>2</sub> + 1 mM MgCL<sub>2</sub> pH 6.0 + 1 mM MnCl<sub>2</sub>. This was followed by desorption with the aid of the same buffer containing 0.5 M methyl- $\alpha$ -D-mannopyranoside. The elution was continuously monitored at 280 nm. The 3 ml fractions that had been collected were assayed for hyaluronidase activity. The active fractions were pooled and dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing sodium azide and 17 mg/ml trehalose. Determination of the concentration of proteins, specific activities of the "pools", and SDS-PAGE analysis was carried out. This step was very effective in removing the rest of hemoglobin. The Con A chromatography resulted in a 4-10 purification factor. This factor differed, depending on the quality of the starting material.

Example 7: - Propyl Fractogel Hydrophobic Interaction Chromatography
To hyaluronidase active Con A-pools ammonium sulfate were added to a final concentration of 2 M. The samples were then incubated 1 h at room temperature with 150 ml Propyl-Fractogel EMD Propyl 650 (S) ®, Merck KgaA, Germany, Art. No. 1.10085, equilibrated with 0.1 M phosphate buffer pH 7.0, containing 2 M ammonium sulfate. After the incubation was finished the gel was washed twice with the same buffer, and the HPLC-Superformance (2.6 cm x 60 cm) column

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was prepared. The bound proteins were eluted with 0.1 M phosphate buffer pH 7.0. The 6 ml fractions were collected every 3 min, directly dialyzed against deionized water (2 - 3 h) and, then against 20 mM phosphate buffer pH 6.0. The fractions were assayed for hyaluronidase activity. The active fractions were pooled and dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing sodium azide and 17 mg/ml trehalose. The protein and activity determination of the pools was carried out.

The purification factor at this chromatography step was about 3 to 5. A small amount of Con A released from the carrier gel in the previous step was removed together with other protein impurities.

Example 8: - Preparation of hyaluronic acid oligosaccharide affinity column Hydrolysis of hyaluronan (HA) with bovine testes hyaluronidase (a) Hyaluronic acid, 7 g was dissolved in 1,25 l of 0.1 M sodium acetate buffer containing 0.15 NaCl and 0.5 mM EDTA, pH 5.2 by mixing overnight at 4°C in the presence of toluene. Thereafter pH of HA containing solution was adjusted to 5.2 and after warming up to 37°C, bovine testes hyaluronidase (Merck KGaA; 700 WHO units/mg) was added. For 7 g of HA, 210 mg of enzyme dissolved immediately before use in 50 ml of the above buffer were used. Hydrolysis was allowed to proceed for 30 min at 37°C with constant stirring, and terminated by heating for 5 min at 100°C in a boiling water bath. The reaction mixture was clarified through centrifugation for 30 min at 10 000 g, denatured protein containing sediment was discarded and supernatant filtered through 0.2 µm filter, on which a glass fiber prefilter was placed. Clarified solution containing HA oligosccharides (HAOS) was fractionated by filtration through tree Diaflo ultrafiltration membrane (Amicon) with different molecular cut off values as follows.

(b) Fractionation of HAOS by ultrafiltration

HAOS-containing solution from the previous step was filtered through 30 YM

Diaflo ultrafiltration membrane. Retentate was saved for other studies while
filtrate was subjected to the second ultrafiltration through 10 YM Diaflo

ultrafiltration membrane. Again, retentate was saved for other studies while the
solution passing through 10 YM was subjected to the last ultrafiltration through 3

YM Diaflo membrane. Thereafter, retentate containing HA-OS, about 10 ml of the

solution, was used for further purification. This fraction: HAOS 3-10 was purified as follows and further used for coupling to Sepharose.

### (c) Purification of HAOS 3-10

HA-OS 3-10 were purified (desalted) on Biogel P2 ® column. This column (4 cm x 100 cm) was packed with Biogel 2 medium ®, 200 – 400 mesh (BioRad), and washed with 5 column volumes of water (Milli Q, Millipore). HAOS 3-10 fraction obtained from the previous step (15 ml; 1.5 g of oligosaccharides) was applied to this column. The column was eluted with water; 15 ml fraction were collected and analyzed for the presence of HA oligosaccharides. Oligosaccharide containing fractions eluted before salts (the latter detected with AgNO3) were combined and concentrated again on 3 YM Diaflo membrane.

# (d) Analysis of HAOS 3 - 10

To determine the coupling efficiency of the Sepharose, gel (the same batch) was washed and suspended in water as to prepare a 50 % slurry. From the

suspension of Sepharose-HAOS 3 – 10 conjugate and Sepharose used as a control, 100 µl aliquots were withdrawn in triplicate and added to 2.5 ml of 2.2 N trifluoroacetic acid (TFA, Merck KgaA) in teflon screw capped tube. For hydrolysis, the mixture were flushed with argon and incubated at 100°C for 16 h. At the end of hydrolysis, samples were dried under nitrogen, re-suspended in water and used for the determination of glucosamine and uronic acid. To determine the extent of uronic acid and glucosamine decomposition for each of the hydrolysis, control samples containing known amounts of UA or GlcNAc were included, and incubated under the same conditions.

Under conditions described above 5, 8, 9, 11 and 15 mg of HAOS 3 – 10 were coupled per 1 ml of drained Sepharose gel in *two independent experiments*. This results are based on the UA and glucosamine assays.

#### (e) Assay used

The content of the uronic acid in the samples analyzed was determined according to Bitter T. and Muir H. M., *Anal. Biochem.*, 1962, 4, 330 – 334.

The hexosamine amounts were analyzed with the method of Rondle C.J.M. and Morgan W.T.J., *Biochem. J.*, 1955, 61, 586 – 593.

<u>Example 9:</u> - Hyaluronic Acid Fragments Sepharose Chromatography (HA-Sepharose Chromatography)

The chromatography matrices containing 8 to 10 mg/ml were prepared as indicated. The enzyme containing sample was dialyzed against 20 mM acetic buffer + 0.15 M NaCl pH 4.0 and applied to the 25 ml HA-Sepharose column. After washing with the same buffer, the elution was done with the 20 mM acetic buffer with a 0.15 to 1 M gradient of NaCl.

The 1 ml fractions were tested in the hyaluronidase-activity determination test, pooled, dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing sodium azide and 17 mg/ml trehalose. The protein and activity determination of the pools was carried out. The purification factor of this chromatography step was about 3.

# Example 10: - Diol-LiChrospher Chromatography

15 A 20 ml active sample dialyzed against Milli-Q-H<sub>2</sub>O was applied on the Diol-LiChrospher column. The column was then equilibrated with 15 ml Milli-Q-H<sub>2</sub>O and washed 5 min with 2 ml water. The elution of the active sample was done 15 min with 20 mM acetic buffer pH 5.9 (gradient, 0 to 5 mM NaCl) and 35 min with gradient 20 mM to100 mM acetic acid buffer pH 5.5 containing 5 mM NaCl. The fractions were assayed for hyaluronidase activity. The active fractions were pooled and dialyzed overnight against 20 mM phosphate buffer pH 6.0 containing sodium azide and 17 mg/ml trehalose. The protein and activity determination of the pools was carried out. The purification factor: 3.

# 25 Example 11: – RP 18e Chromatography

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This purification step can be used only as the last one and is aimed to obtain the sample devoid of salts and other protein impurities (e. g. peptide protease inhibitors). The hyaluronidase activity was completely lost, because manillase is not resistance to organic solvents used in this step. Manillase sample was applied to the RP 18e column. The 0.25 ml/min fractions were collected. The elution was done in the presence of 0.1% TFA and, gradient water to 99% of acetonitrile was used. The RP-purified samples can be used directly for amino acid sequencing, MALDI measurement, carbohydrate structure analysis and as standard for purification of other batches of manillase.

#### Example 12: - Activity Determination - Turbidity Reduction Test

The hyaluronidase activity determination was done with the turbidity reduction measurements. Commercially available preparations of hyaluronan (isolated from the different animal tissues and fluids, e.g. human cord, rooster comb) and

hyaluronidases (endo-ß-glucosaminidases from bovine testes, porcine testes, bee venom; lyases from Streptomyces hyalurolyticus) were used for establishing suitable activity assay conditions. The endo-ß-glucuronidase from Hirudo medicinalis was partially purified in our laboratory.

Hyaluronan stock solution (conc. 2 mg/ml) was prepared by dissolving HA in 0.3

M phosphate buffer pH 5.3. This solution was diluted with the same buffer to a concentration of 0.2 mg/ml directly before the test. The enzyme-containing samples were diluted to an appropriate amount of enzyme (0.5 - 5 WHO units) with 20 mM phosphate buffer containing 0.01% of bovine albumin and 77mM of NaCl (enzyme dilution buffer). To 0.1 ml of these samples, 0.1 ml hyaluronan (0.2 mg/ml) solution was added, mixed and incubated 45 minutes at 37°C. The test was done in duplicate. The reaction was stopped by dilution with 1.0 ml of

was done in duplicate. The reaction was stopped by dilution with 1.0 ml of albumin reagent (0.1% of albumin dissolved in 80 mM acetic acid/ 40 mM sodium acetate buffer, pH 3.75). After 10 min incubation at RT or 37°C the optical density at 600 nm was read and the activity was expressed in WHO (IU) units by comparison (SLT-program) with a standard. The WHO preparation of bovine testicular hyaluronidase (Humphrey J. H., Bull. World Health Org. 1957, 16, 291-

#### Example 13: - Protein Estimation

294) was used as standard.

The protein content of column eluents was determined by measuring the ultraviolet absorbance of solutions at 280 nm. The protein concentration of the pooled fractions was determined with the aid of Pierce micromethod. The BSA solution was used as a reference protein.

### 30 Example 14: - SDS-PAGE Electrophoresis

Electrophoresis was done according to Laemmli procedure (Nature, 1970, 227, 680-685). The following gels were used: 4 to 20% gradient or 12,5% separating gels with 4% stacking gel. Samples were subjected to electrophoresis in the presence of sodium dodecyl sulfate and ß-mercaptoethanol. Proteins were

visualized after staining with Coomassie brilliant blue and/or Silver staining (according to Pharmacia instruction).

#### Example 15: - Isoelectric Focusing

- To pursue isoelectric focusing studies on the manillase preparation, the protocol provided by supplier (Pharmacia) was adopted. Following focusing, the gel was fixed and silver stained (according to Pharmacia protocol).
- Example 16: Preparation of Immunoglobin from Immune Sera of Rabbits

  (anti-ConA, anti-hemoglobin and anti-peptide rabbit antibodies)

  The rabbit sera were raised with the use of the following immunogens:

  concanavalin A lectin, mixture of hemoglobins and peptide-KLH conjugates. The peptide sequence was identical with that of the 14 amino acid N-terminal part of manillase (KEIAVTIDDKNVIA).
- The sera were purified on the Protein A Sepharose (Pharmacia, 17-0780-01) column according to the standard Pharmacia instruction. The purity of the IgG samples were checked with the aid of SDS-PAGE and ELISA-test.

#### Example 17: - Western-Immunoblot Assay

Suitable aliquots of the samples and pre-stained protein marker of known molecular weight were subjected to SDS-PAGE as described above. A pre-stained BioRad molecular weight marker was used. The protein was transferred electrophoretically from polyacrylamide gels (0,8 mA/cm2) to immobile polyvinyldifluoride (PVDF) membranes in the presence of transfer buffer for 100 min. The PVDF membrane was incubated with blocking solution (PBS, pH 7.5 + 2% fat free milk) for 1 h at room temperature. Next, the membrane was incubated 2 h at room temperature with the antibody, appropriately diluted with the blocking solution. The membrane was washed with TBS+0.05% Tween 20, pH 7.5 and incubated for 2 h at room temperature with (a second antibody) goat anti-rabbit-alkaline phosphate conjugate, BioRad. The membrane was washed two times with TBS+Tween 20 and incubated 10 min with BCIP alkaline phosphatase substrate solution. Adding a stopping buffer terminated the reaction.

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#### Example 18: - Amino Acid Sequencing

The sequence of N-terminal 33 amino acid residues of the manillase was obtained by Edman degradation. After SDS-PAGE of manillase-active samples, the bands were transferred onto PDVF membrane, stained with Coomassie Blue, cut-out and sequenced. The same amino sequence was found for the sample obtained after the last purification step with the aid of RP-column chromatography.

#### Example 19: - pH Dependence of Enzyme Activity

(for hyaluronidase isolated from Hirudinaria manillensis and Hirudo medicinalis leech heads)

Samples of hyaluronidase used in this experiment were extracted either from Hirudinaria manillensis or Hirudo medicinalis leech heads and partially purified with the aid of ammonium sulfate precipitation and cation exchange

chromatography. Each sample containing 500 WHO units/ml was incubated at - 20°C, +4°C and 37°C at a range of pHs from 2.6 to 9.0 (20 mM acetic for pH 2.6 to 5; 20 mM phosphate buffer for pH 5 to 9). The enzyme activity was measured after 1, 2 and 7 days incubation periods. At both acid and alkaline extremes of pH, inhibition of activity to the same extent was observed for both hyaluronidases.

However, during longer incubation periods manillase was more stable then Hirudo medicinalis hyaluronidase: e.g. after 7 days incubation at pH 7.0 at +4°C and 37°C - manillase retained 75% and 60% of the starting activity, respectively. The Hirudo medicinalis hyaluronidase incubated at the same conditions was already inactive after 1 day.

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Example 20: - Stability Measurement of Hyaluronidases in the Presence of Dog's Serum (for hyaluronidase isolated from Hirudinaria manillensis and Hirudo medicinalis leech heads)

The 5 kU/ml samples of manillase, Hirudo medicinalis and bovine testes hyaluronidase were diluted with dog's or rat's citrated plasma to a final concentration of 250 U/ml. Next, these solutions were incubated at -20°C, + 4°C and +37°C for 0 to 7 days. The controls containing the same hyaluronidases, diluted in buffer were included in this experiment. Finally, the hyaluronidase activity was measured.

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#### Example 21: - Contaminating Enzyme Activities

At each stage of the purification procedure for leech hyaluronidase, the preparation was checked for other enzymes capable of degrading protein with the aid of universal protease substrate (Boehringer Mannheim, cat. no. 1080 733) according to Twining S. S. (Anal. Biochem., 1984, 143, 30-34).

# Cleavage of a hyaluronan by hyaluronidases results in the liberation of reducing sugars. The amount of the liberated sugars was measured colorimetrically by the modified method of Park (Park J. & Johnson M.: J. Biol. Chem. 1949, 181, 149)

Example 22: - Influence of Heparin on Hyaluronidase Activity

modified method of Park (Park J. & Johnson M.; J. Biol. Chem. 1949, 181, 149). For the measurement of the influence of heparin on the activity of manillase and bovine testes hyaluronidase, two activity determination were carried out: one in the presence of heparin, and second without heparin. Hyaluronidase samples, 25  $\mu$ l (3.2 WHO units) were incubated 30 min at 37°C with 25  $\mu$ l of the heparin (Liquemin, Fa. Hoffmann LaRoche) solution, containing 0 to 24 units of heparin.

Then, 50 µl of hyaluronan (2.5 mg/ml) was added and the incubation was continued for 30 min at 37°C. The reaction was terminated by heating for 2 min at 100°C. Next, 100 µl of carbonate-cyanide solution and 100 µl of potassium ferricyanide solution were added to the inactivated digest. The samples were heated in a boiling water bath for 15 min and then cooled in an ice bath.

Afterwards, 0.75  $\mu$ l of ferric ammonium sulfate solution was added to the reaction mixtures. After 15 min incubation at RT, the color developed was measured at 690 nm in a Shimadzu spectrophotometer. Suitable blanks and no-enzyme controls were included in each assay. The expected reducing sugar (glucuronic acid or N-acetyl-glucosamine, 1 to 15  $\mu$ g) for the type of sample under analysis was used as standard.

### Example 23: - Deglycosylation of the Manillase

The samples of manillase were deglycosylated with the aid of PNGase F enzyme (BioLabs Art. No. 701 L) according to supplier instruction. The deglycosylation was done under denaturing and native conditions. The O-glycanase, neuraminidase and neuraminidase + O-glycanase treatments were done according to Boehringer Mannheim standard prescriptions. All samples were characterized with the SDS-PAGE and activity determination test.

Example 24: - Construction of the E. coli Expression Vector (Fig. 11)
For expression in E. coli we used a modified version of the plasmid pASK75,
which carries the tet promoter region. {Skerra, Gene 151, (1994), pp 131-135 }.

The modification we made by cloning a new linker between the Xbal an Hind III sites. The new linker contains the ompA leader sequence, another multiple cloning site and a 6xHis-tag instead of the strep-tag.

Linkersequence which was cloned in pASK75.

- To construct the expression vector for manillase it was necessary to introduce 5'
  Cla I and 3' Eco47III restriction sites by PCR method. Therefore the two primers
  - 5' ATC GAT AAA GAG ATT GCC GTG AC and
  - 3' GTT GTT TCC GAT GCT AAA GCG CT

were used. The PCR product first was cloned into the PCR II vector system (Invitrogen) and sequenced.

In a second step the manillase gene was cloned into the modified pASK75 vector using the rectrictionsites 5'Clal and 3' Eco47III.

After expressing and proving the activity of this recombinant manillase in a second PCR reaction the His-tag was removed and the start codon of the manillase gene was directly fused to the omp A leader sequence. The primers for this PCR reaction were:

- 5' ACC GTA GCG CAG GCC AAA GAG ATT GCC GTG and
- 3' CAC GGC AAT CTC TTT GGC CTG CGC TAC GGT.
- Example 25: Construction of the Baculo Donor Plasmid (Fig. 12)

  For expression of manillase in the Baculo virus expression system the Bac-To-Bac<sup>™</sup> Baculovirus Expression System from Gibco Life Technologies was used.

  To get a section system the Honeybee melitin leader sequence was fused to the

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manillase gene and to introduce the restriction sites 5' BamHI and 3' KpnI one single PCR reaction was carried out.

#### 5'Primer:

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CGG ATC CAT GAA ATT CTT AGT CAA CGT TGC CCT TGT TTT TAT GGT

CGT ATA CAT TTC TTA CAT CTA TGC GAA AGA GAT TGC CGT GAC

3' Primer:

AAT GTT GAA GCA TAA GGT ACC

The PCR product was cloned into the PCR II Vector (Invitrogen) and sequenced.

Then the Melitin – Manillase Fusion was cloned into the pFastBac vector using the restriction sites 5'BamHI and 3'KpnI (Fig. 12).

Example 26: - Construction of the Yeast Expression Vector (Fig. 13)

For expression in yeast we used the pichia multi copy expression system
(Invitrogen). To construct the expression vector for manillase we used the PCR amplification method of the manillase gene in such a way that compatible restriction ends (5´ EcoR I, 3´Not I) are generated for ligation into the appropriate vector (pPIC9K). Therefore the following primers were used:

- 5' GTA GAA TTC AAA GAG ATT GCC GTG ACA
- 3' GAT GCT AAT GTT GAA GCA TAA TGA GCG GCC GC
- 20 Before transforming the Pichia Speroplasts the expression vector has to be liniarized with Sal I.

#### Example 26: - Expression in E. coli

In the expression vector pRG72, which contains the structural gene of Sarastatin fused to the ompA leader sequence, was transformed into W3110 competent cells. The cells were grown to a mid-log phase, and the promoter was then induced by adding 200µg aTC / I. 1 h thereafter the recombinant manillase could be clearly detected.

Example 27: - Generation of Recombinant Baculoviruses and Manillase

Expression with the Bac-To-Bac Expression System

The donor plasmid pTD13 was transformed into DH10Bac competent cells which contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the donor plasmid ca transpose to the a mini-attTn7 target

site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids were identified by disruption of the *lacZ* gene. High molecular weight mini-prep DNA prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA was then used to transfect insect cells.

Detailed description could be find in the instruction manual of the expression kit.

#### Example 28: - Expression in yeast

To be sure to have integrated the manillase gene the colonies have to be screened for His<sup>+</sup> Mut<sup>+</sup>-mutants

Using a single colony, inoculate 100 ml Medium i a 1 l flask. Growing conditions are: 28 – 30°C, 250 rpm, up to OD 2-6. To induce expression, first cetrifuge the culture, decant to supernatant and re-suspend the cell pellet in new medium using 1/5 of the original culture volume. Add 100% methanol to a final concentration of 0,5% every 24 hours to maintain induction. After max 6 days supernatant is analyzed by SDS-Page and the activity assay.

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#### Patent Claims

- 1. A purified protein isolated from the leech species *Hirudinaria manillensis* having the biological activity of a hyaluronidase which is not influenced in its activity by heparin, characterized in that it has a molecular weight of 53 60 dependent on glycosylation.
- 2. A glycosylated protein according to claim 1 having a molecular weight of 58 (±2).
- 3. A non-glycosylated protein according to claim 1 having a molecular weight of 54 ( $\pm$ 2).
- 4. A protein according to any of claims 1-3 having an isoelectric point of 7.2-8.0.
  - A protein according to any of claims 1 4 having the amino acid sequence given in Fig. 7 and SEQ ID No. 1.
- 20 6. A protein according to claims 1 5 having a specific enzymatic activity of > 100 kU / mg protein.
  - A process for isolating and purifying the protein as defined in claims 1 6
     comprising the following steps
- 25 (i) homogenization of heads of leeches of the species *Hirudinaria* manillensis with an acid buffer and centrifugation,
  - (ii) ammonium sulfate precipitation of the supernatant of step (i),
  - (iii) cation exchange chromatography,
  - (iv) concanavalin A affinity chromatography
  - (v) hydrophobic interaction chromatography
    - (vi) affinity chromatography on matrices coated with hyaluronic acid fragments
    - (vii) gel permeation chromatography, and optionally
    - (viii) enzymatic or chemical de-glycosylation of the purified protein.

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- 8. A protein having the biological activity of a hyaluronidase which is not influenced in its activity by heparin and having a molecular weight of 53 60 dependent on glycosylation, obtainable by the process steps of claim 7.

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- A protein according to claim 8 having a specific enzymatic activity of > 100
   kU / mg protein.
- 10. A DNA sequence coding for a protein of claim 1 and 9.
  - 11. A DNA sequence coding for a protein of claim 8 comprising any nucleotide sequence depicted in Fig. 8 (SEQ. ID No. 2), Fig. 9 (SEQ. ID No. 4) and Fig.10 (SEQ ID No. 6).
  - 12. A recombinant protein having the biological activity of a hyaluronidase encoded by any a DNA sequence of claim 11.
- 13. A recombinant protein with the biological activity of a hyaluronidase and a molecular weight of 55 59 dependent on glycosylation having any amino acid sequence depicted in Fig. 8, 9 and 10 (SEQ. ID Nos. 3, 5, 7) or a sequence which has a homology to said sequences of at least 80%.
  - 14. An expression vector comprising a DNA sequence of claim 10 or 11.
  - 15. A host cell suitable for the expression of a protein of claim 12 or 13 which was transformed with a vector of claim 14.
- 16. A protein according to any of claims 1 6, 8, 9, 12 and 13 as a medicament.
  - 17. A pharmaceutical composition comprising the protein of claim 16 and a pharmaceutically acceptable diluent, carrier or excipient therefor.

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- 18. A pharmaceutical composition comprising additionally a pharmacologically active compound.
- 19. A pharmaceutical composition according to claim 18, wherein the pharmacological active compound is heparin.
- 20. The use of a protein according to any of claims 1 6, 8, 9, 12 and 13 in the manufacture of a medicament for treating myocardial, cardiovascular and thrombotic disorders and tumors.

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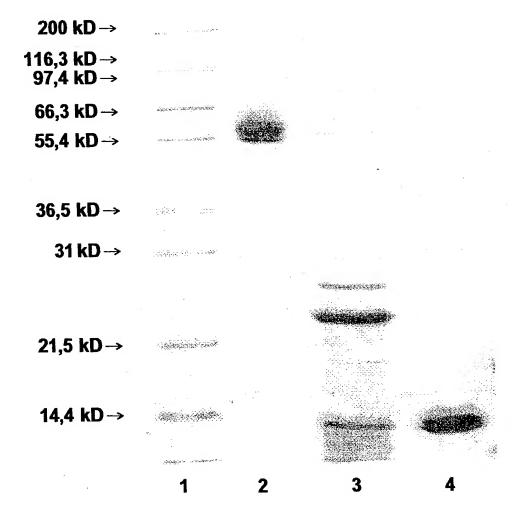
(54) Title: HYALURONIDASE FROM THE HIRUDINARIA MANILLENSIS, ISOLATION, PURIFICATION AND RECOMBINANT METHOD OF PRODUCTION

(57) Abstract: The present invention relates to the isolation, purification and characterization of a hyaluronidase which derives from the tropical leech *Hirudinaria manillensis*. Therefore, according to this invention, the enzyme was called "manillase". The invention is furthermore concerned with the recombinant method of production of manillase which includes the disclosure of DNA and amino acid sequences as well as of expression vectors and host systems. Finally, the invention relates to the use of manillase for therapeutic purposes, for example, for the treatment of myocardial diseases, thrombotic events and tumors.

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# Fig.1

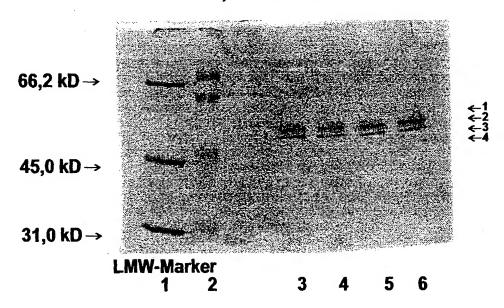


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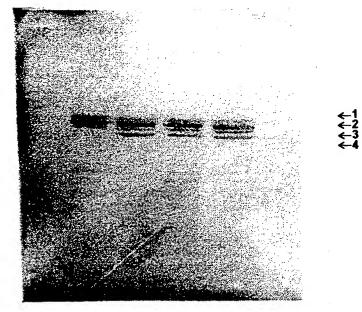
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Fig.2





# b) - SDS-PAGE-Western blot



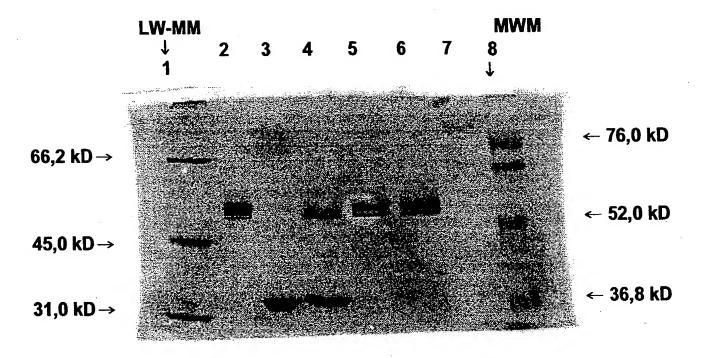
LMW-Marker 1 3

SUBSTITUTE SHEET (RULE 26)

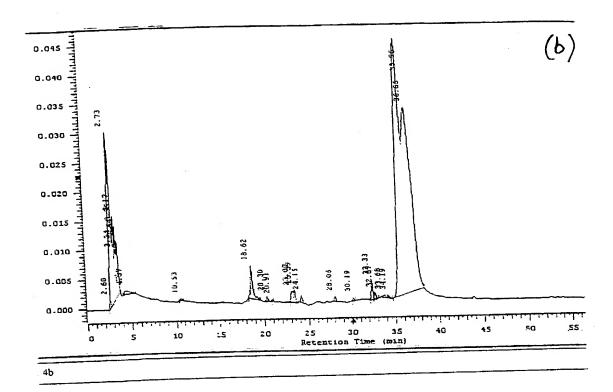
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Fig.3



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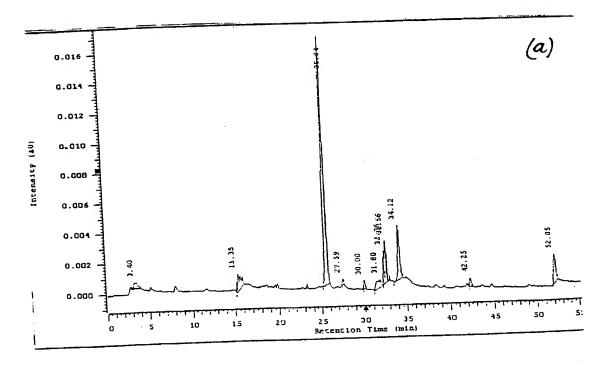


Fig. 4

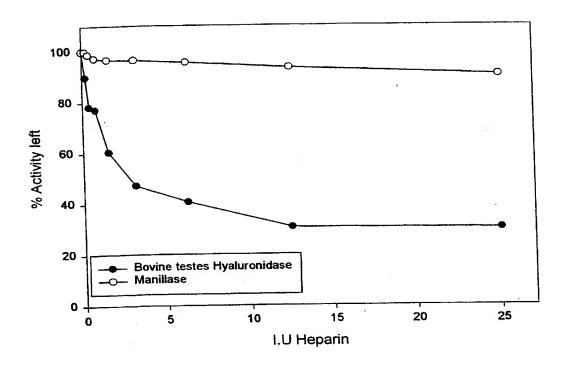
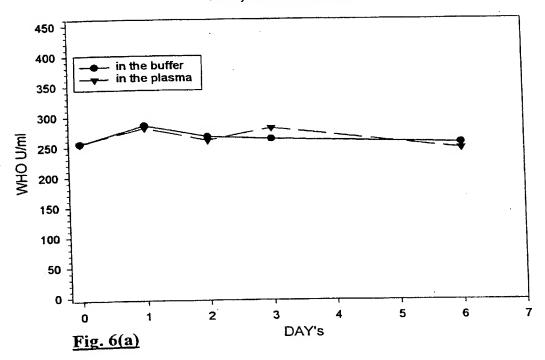


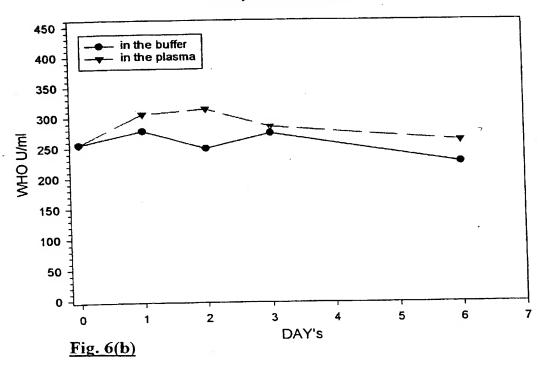
Fig.5

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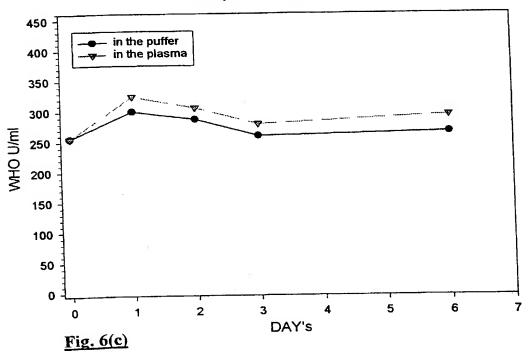
activity of Manillase at 4°C



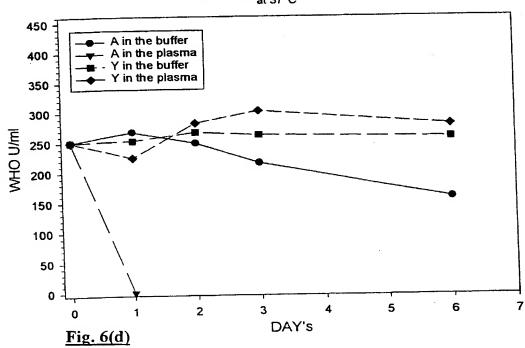
### activity of Manillase at -20°C







activity of
Hirudo medicinalis hyaluronidase (A) and
bovine testes hyaluronidase (Y)
at 37°C



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# Fig. 7

KEIAVTIDDK	NVIASVSESF	HGVAFDASLF	SPKGLWSFVD	ITSPKLFKLL	50
EGLSPGYFRV	GGTFANWLFF	DLDENNKWKD	YWAFKDKTPE	TATITRRWLF	100
RKQNNLKKET	EDDLVKLTKG	SKMRLLFDLN	AEVRTGYEIG	KKMTSTWDSS	150
EAEKLFKYCV	SKGYGDNIDW	ELGNEPDHTS	AHNLTEKQVG	EDFKALHKVL	200
EKYPTLNKGS	LVGPDVGWMG	VSYVKGLADG	AGDLVTAFTL	HQYYFDGNTS	250
DVSTYLDATY	FKKLQQLFDK	VKDVLKNSQH	KDKPLWLGET	SSGYNSGTKD	300
VSDRYVSGFL	TLDKLGLSAA	NNVKVVIRQT	IYNGYYGLLD	KNTLEPNPDY	350
WLMHVHNSLV	GNTVFKVDVS	DPTNKARVYA	QCTKTNSKHT	QSRYYKGSLT	400
IFALNVGDED	VTLKIDQYGG	KKIYSYILTP	EGGQLTSQKV	LLNGKELKLV	450
SDOLPELNAN	ESKTSFTLSP	KTFGFFVVSD	ANVEACKK		488

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# Fig. 8:

											*
AAA	GAG	TTA	GCC	GTG	ACA	ATT	GAC	GAT	AAG		GTG
K	E	I	A	V	T	I	D	D	K		V
ATT	GCA	TCT	GCC	AGT	GGG	TCT	TTC	CTT	GGA	GTT	GCC
I	A	S	A	S	G	S	F	L	G	V	A
TTT	GAT	GCG	TCT	CTA	TTT	TCG	CCC	AAG	GGT	CTT	TGG
F	D	A	S	L	F	S	P	K	G	L	W
AGC	TTT	GTT	GAT	ATT	ACC	TCT	CCA	AAA	TTG	TTC	AAA
S	F	V	D	I	T	S	P	K	L	F	K
TTG	CTG	GAA	GGA	CTT	TCT	CCT	GGA	TAC	TTC	AGG	GTT
L	L	E	G	L	S	P	G	Y	F	R	V
GGC	GGA	ACG	TTT	GCC	AAT	TGG	CTG	TTT	TTT	GAC	TTG
G	G	T	F	A	N	W	L	F	F	D	L
GAC	GAA	AAT	AAT	AAG	TGG	AAG	GAT	TAT	TGG	GCT	TTT
D	E	N	N	K	W	K	D	Y	W	A	F
AAA	GAC	AAA	ACC	CCC	GAA	ACT	GCG	ACA	ATA	ACA	AGG
K	D	K	T	P	E	T	A	T	I	T	R
AGA	. TGG	CTG	TTC	AGA	AAA	CAA	AAT	AAT	CTG	AAA	AAG
R	W	L	F	R	K	Q	N	N	L	K	K
GAG	ACT	TTT	GAC	AAT	TTA	GTG	AAA	CTA	ACA	AAG	GGA
E	T	F	D	N	L	V	K	L	T	K	G
AGC	AAG	ATG	AGA	TTG	TTA	TTC	GAT	TTG	AAT	GCC	GAA
S	K	M	R	L	L	F	D	L	N	A	E
GTG	AGG	ACT	GGT	TAT	GAA	ATT	GGA	AAG	AAG	ATG	ACA
V	R	T	G	Y	E	I	G	K	K	M	T
TCC	ACT	TGG	GAT	TCA	TCG	GAG	GCT	GAA	AAG	TTA	TTT
S	T	W	D	S	S	E	A	E	K	L	F
AAA K	TAT	_	GTG V	TCA S		GGT G	TAC Y	GGA G	GAC D	AAT N	ATC I
GAT	TGG	GAA	CTT	GGA	AAT	GAA	CCG	GAC	CAC	ACC	TCA
D	W	E	L	G	N	E	P	D	H	T	S
GCT	CAC	AAT	TTA	ACT	GAA	AAG	CAG	GTT	GGA	GAA	GAT
A		N	L	T	E	K	Q	V	G	E	D
TTI F	T AAF	A GCA	CTG	CAT H	AAA K	GTT V	CTA L	GAG E	AAA K	TAT Y	CCA P

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# Fig 8 (contnd)

ACT	CTT	AAC	AAG	GGA	TCG	CTC	GTT	GGT	CCA	GAT	GTA
T	L	N	K	G	S	L	V	G	P	D	V
GGG	TGG	ATG	GGC	GTC	AGT	WAC	GTC	AAG	GGA	TTG	GCA
G	W	M	G	V	S	Y	V	K	G	L	A
GAC	GAG	GCR	GGT	GAC	CAT	GTA	ACK	GCT A	TTT	ACA	CTC
D	E	A	G	D	H	V	T		F	T	L
CAC	CAA	TAT	TAT	TTC	GAT	GGA	AAC	ACY	TCT	GAT	gta
H	Q	Y	Y	F	D	G	N	T	S	D	V
TCA	ATA	TAT	CTT	GAT	GCC	ACA	TAC	TTT	AAG	<b>AA</b> G	CTG
S	I	Y	L	D	A	T	Y	F	K	K	L
CAA	CAA	CTA	TTT	GAT	AAA	GTG	AAA	GAT	GTT	TTG	AAA
Q	Q	L	F	D	K	V	K	D	V	L	K
GAT	TCT	CCA	CAT	AAA	GAC	GAA	CCA	TTA	TGG	CTT	GGA
D	S	P	H	K	D	E	P	L	W	L	G
GAA	ACA	AGT	TCT	GGA	TAC	AAC	AGC	GGC	ACA	GAA	GAT
E	T	S	S	G	Y	N	S	G	T	E	D
GTA	TĊC	GAT	CGA	TAT	GTT	TCA	GGA	TTT	CTA	ACA	TTA
V	S	D	R	Y	V	S	G	F	L	T	L
GAC	AAG	TTG	GGT	CTC ·	agt	GCA	GCC	AAC	AAT	GTA	AAG
D	K	L	G		s	A	A	N	N	V	K
G <b>T</b> T	GTT	ATA	AGA	CAG	ACA	ATA	TAC	AAT	GGA	TAT	TAT
V	V	I	R	Q	T	I	Y	N	G	Y	Y
GGT	CTC	CTT	GAC	AAA	AAC	ACT	TTA	GAG	CCG	AAT	CCG
G	L	L	D	K	N	T	L	E	P	N	P
GAT	TAC	TGG	TTA	ATG	CAT	GTT	CAT	AAT	TCT	TTG	GTC
D		W	L	M	H	V	H	N	S	L	V
GGA	AAT	ACA	GTT	TTT	AAA	GTT	GAC	GTT	AGT	GAT	CCA
G	N	T	V	F	K	V	D	V	S	D	P
ACT	AAT	AAA	GCA	AGA	GTT	TAC	GCG	CAA	TGT	ACC	AAA
T	N	K	A	R	V	Y	A	Q	C	T	K
ACA	AAT	AGC	AAA	CAT	ACT	CAA	AGC	AGA	TAT	TAC	aag
T	N	S	K	H	T	Q	S	R	Y	Y	K
GGC G		TTG L	ACA T	ATC I	TTT F	GCA A	CTT L	AAT N	GTT V	G GGA	GAT D

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## Fig 8 (contnd)

GGA	GAT	GTA	ACG	TTA	AAG	ATC	GGT	CAA	TAC	AGC	GGT
G	D	V	T	L	K	I	G	Q	Y	S	G
AAA	AAA	ATT	TAT	TCA	TAC	ATT I	CTG	ACA	CCT	GAA	GGA
K	K	I	Y	S	Y		L	T	P	E	G
GGA	CAA	CTT	ACA	TCA	CAG	AAA	GTT	CTC	TTG	AAT	GGA
G	Q	L	T	S	Q	K		L	L	N	G
AAG	GAA	TTG	AAC	TTA	GTG	TCT	GAT	CAG	TTA	CCA	GAA
K	E	L	N	L	V	S	D	Q	L	P	E
CTA	AAT	GCA	GAT	GAA	TCC	AAA	ACA	TCT	TTC	ACC	TTA
L	N	A	D	E	S	K	T	S	F	T	L
TCC	CCA	AAG	ACA	TTT	GGT	TTT	TTT	GTT	GTT	TCC	GAT
S	P	K	T	F	G	F	F	V	V	S	D
GCT A	AAT N	GTT V	GAA E	GCA A	TGY C	AAR K	AAR K				

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## Fig. 9:

AAA K	GAG E	TTA I	GC A	C GI		ACA T	TTA I	GAC D	G <i>P</i> D		AAG K	TAA N	GTG V
ATT I	GCA A	TCT S	GC A	C A		GAG E	TCT S	TTC F	C CI		GGA G	GTT V >	GCC A
TTT F	GAT D	GCG A	TC S	T C		TTT F	TCG S	CC( P	C A		GGT G	CTT L	TGG W
AGC S	TTT F	GTT V	r G <i>I</i> D	AT A	TT.	ACC T	TCT S	CC. P	A A	AA C	TTG L	TTC F	AAA K
TTG L	CTG L	GA E	A G		TT	TCT S	CCT P	GG G		TAC (	TTC F	AGG R	GTT V
GGC G	GGA G	AC T	G T		GCC A	AAT N	CGG R	C]	_	ITT F	TTT F	GAC D	TTG L
GAC D	GA <i>F</i> E	A.A N	A T.		aag K	TGG W	AAR K	G2 D		TAT Y	TGG W	GCT A	TTT F
AAA K	GA(	C AF			CCC P	GAA E	ACT T	G A		ACA T	ATA I	ACA T	AGG R
AGA R	TG W	G C		rtc f	AGA R	AAA K	CAA Q	. A N		AAT N	CTG L	AAA K	AAG K
GAG E	, AC	T T'		GAC D	AAT N	TTA L	GT(		AA <	CTA L	ACA T	AAG K	GGA G
AGC S	: AF	AG A	_	AGA R	TTG L	TTA L	TT(		TAE D	TTG L	AAT N	GCC A	GAA E
GT(	g AC R	_		GGT G	TAT Y	GAA E	AT'	_	GGA G	AAG K	AAG K	ATC M	G ACA T
TC(	C A	-	:GG I	GAT D	TCA S	TC0	G GA E	_	GCT A	GAA E	AA( K		A TTT F
AA K	A T Y		rgt C	GTG V	TCA S	AA K	A GG G	T	TAC Y	GG <i>P</i> G	A GA		
GA D	T T	'GG	GAA E	CTT L	GGG	AA N		SΑ	CCG P	GA(	C CA H	C AC	C TCA
G( A	CT C	CAC	AAT N	TTA L	AC:	r GA E	A A	AG	CAG Q	GT V	T GO	_	AA GAT D
	rr 2	AAA K	GCA A	CTG L	CA'	T AA K	AA G V	TT	CTA L	GA E	.G A <i>l</i> K		AT CCA P
	СТ	CTT L	AAC N	AAG K	GG G	A TO	CG C	TC	GTT V		GT C		AT GTA V

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### Fig 9 (contnd)

				•							
GGG G	TGG W	ATG M	GGC G	GTC V	AGT S	TAC Y	GTC V	AAG K	GGA G		GCA A
GAC	GAG	GCA	GGT	GAC	CAT	GTA	ACT	GCT	TTT	ACA	CTC
D	E	A	G	D	H	V	T	A	F	T	L
CAC	CAA	TAT	TAT	TTC	GAT	GGA	AAC	ACC	TCT	GAT	GTA
H	Q	Y	Y	F	D	G	N	T	S	D	V
TCA	ATA	TAT	CTT	GAT	GCC	ACA	TAC	TTT	AAG	AAG	CTG
S	I	Y	L	D	A	T	Y	F	K	K	L
CAA	CAA	CTA	TTT	GAT	AAA	GTG	AAA	GAT	GTT	TTG	AAA
Q	Q	L	F	D	K	V	K	D	V	L	K
GAT	TCT	CCA	CAT	AAA	GAC	AAA	CCA	TTA	TGG	CTT	GGA
D	S	P	H	K	D	K	P	L	W	L	G
GAA	ACA	AGT	TCT	GGA	TAC	AAC	AGC	GGC	ACA	GAA	GAT
E	T	S	S	G	Y	N	S	G	T	E	D
GTA	TCC	GAT	CGA	TAT	GTT	TCA	GGA	TTT	CTA	ACA	TTA
V	S	D	R	Y	V	S	G	F	L	T	L
GAC	AAG	TTG	GGT	CTC	AGT	GCA	GCC	AAC	AAT	GTA	AAG
D	K	L	G	L	S	A	A	N	N	V	K
GTT	G <b>T</b> T	ATA	AGA	CAG	ACA	ATA	TAC	AGT	GGA	TAT	TAT
V	V	I	R	Q	T	I	Y	S	G	Y	Y
GGT	CCC	CTT	GAC	AAA	AAC	ACT	TTA	GAG	CCA	AAT	CCG
G	P	L	D	K	N	T	L	E	P	N	P
GAT	TAC	TGG	TTA	ATG	CAT	GTT	CAT	AAT	TCT	<b>TT</b> G	GTC
D	Y	W	L	M	H	V	H	N	S	L	V
GGA	AAT	ACA	GTT	TTT	AAA	GTT	GAC	GTT	AGT	GAT	CCA
G	N	T	V	F	K	V	D	V	S	D	P
ACT	AAT	AAA	GCA	AGA	GTT	TAC	GCG	CAA	TGT	ACC	AAA
T	N	K	A	R	V	Y	A	Q	C	T	K
ACA T	AAT N	AGC S	AAA K	CAT H	ACT T	CAA Q	AGC S	AGA R	TAT Y		AAG K
GGC G		TTG	ACA T	ATC I	TTT F	GCA A	CTT L	' AAT N	GTT V	G GGA	GAT D
GA <i>P</i> E			ACG T	TTA L	AAG K	ATC I	G GGT	CAA	Y TAC	AGC S	GGT G

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## Fig 9 (contnd)

AAA	AAA	ATT	TAT	TCA	TAC	ATT	CTG	ACA	CCT	GAA	GGA
K	K	I	Y	S	Y	I	L	T	P	E	G
GGA	CAA	CTT	ACA	TCA	CAG	AAA	GTT	CTC	TTG	AAT	GGA
G	Q	L	T	S	Q	K	V	L	L	N	G
AAG	GAA	TTG	AAC	TTA	RTG	TCT	GAT	CAG	TTA	CCA	CAA.
K	E	L	N	L	V	S	D	Q	L	P	Q
CTA	AAT	GCA	YAT	GAA	TCC	AAA	ACA	TCT	TTC	ACC	TTA
L	N	A	D	E	S	K	T	S	F	T	L
TCC	CCA	AAG	ACA	TTT .	GGT	TTT	TTT	GTT	GTT	TCC	GAT
S	P	K	T		G	F	F	V	V	S	D
GCT	AAT	GTT	GAA	GCA	TGY	AAR	AAR				
A	N	V	E	A	С	K	K				

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## Fig. 10:

AAA	GAG	ATT	GCC	GTG	ACA	ATT	GAC	GAT	AAG	AAT	GTG
K	E	I	A	V	T	I	D	D	K	N	V
ATT	GCA	TCT	GTC	AGT	GAG	TCT	TTC	CAT	GGA	GTT	GCC
I	A	S	V	S	E	S	F		G	V	A
TTT	GAT	GCG	TCT	CTA	TTC	TCG	CCC	AAG	GGT	CCT	TGG
F	D	A	S	L	F	S	P	K	G	P	W
AGC	TTT	GTT	AAT	ATT	ACC	TCT	CCA	AAA	TTG	TTC	AAA
S	F	V	N	I	T	S	P	K	L	F	K
TTG	CTG	GAA	GGA	CTT	TCT	CCT	GGA	TAC	TTC	AGG	GTT
L	L	E	G	L	S	P	G	Y	F	R	V
GGC	GGA	ACG	TTT	GCC	AAT	TGG	CTG	TTT	TTT	GAC	TTG
G	G	T	F	A	N	W	L	F	F	D	L
GAC	GAA	AAT	AAT	AAG	TGG	AAG	GAT	TAT	TGG	GCT	TTT
D	E	N	N	K	W	K	D	Y	W	A	F
AAA	GAC	AAA	ACC	CCC	GAA	ACT	GCG	ACA	ATA	ACA	AGG
K	D	K	T	P	E	T	A	T	I	T	R
AGA	TGG	CTG	TTC	AGA	AAA	CAA	AAT	AAT	CTG	AAA	AAG
R	W	L	F	R	K	Q	N	N	L	K	K
GAG	ACT	TTT	GAC	GAT	TTA	GTG	AAA	CTA	ACA	AAG	GGA
E	T	F	D	D	L	V	K	L	T	K	G
AGC	AAG	ATG	AGA	TTG	TTA	TTC	GAT	TTG	AAT	GCC	GAA
S	K	M	R	L	L	F	D	L	N	A	E
GTG	AGG	ACT	GGT	TAT	GAA	ATT	GGA	AAG	AAG	ACG	ACA
V	R	T	G	Y	E	I	G	K	K	T	T
TCC	ACT	TGG	GAT	TCA	TCG	GAG	GCT	GAA	AAG	TTA	TTT
	T	W	D	S	S	E	A	E	K	L	F
AAA	TAT	TGT	GTG	TCA	AAA	GGT	TAC	GGA	GAC	TAA	ATC
K	Y	C	V	S	K	G	Y	G	D	N	I
GAT	TGG	GAA	CTT	GGA	AAT	GAA	CCG	GAC	CAC	ACC	TCA
D	W	E	L	G	N	E	P	D	H	T	S
GCT	CAC	AAT	TTA	ACT	GAA	AAG	CAG	GTT	GGA	GAA	GAT
A	H	N	L	T	E	K	Q	V	G	E	D
TTC	AAA	GCA	CTG	CAT	AAA	GTT	TTA	GAG	AAA	TAT	CCA
F	K	A	L	H	K	V	L	E	K	Y	P

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## Fig 10 (contnd)

ACT	CTT	AAC	AAG	GGA	TCG	CCC	GTT	GGT	CCA	GAT	GTA
T	L	N	K	G	S	P	V	G	P	D	V
GGG	TGG	ATG	GGC	GTC	AGC	TAC	GTC	AAG	GGA	TTG	GCA
G	W	M	G	V	S	Y	V	K	G	L	A
GAC	GGG	GCA	GGT	GAC	CTT	GTA	ACT	GCT	TTT	ACA	CTA
D	G	A	G	D	L	V	T	A	F	T	L
CAC	CAA	TAT	TAT	TTC	GAT	GGA	AAC	ACC	TCT	GAT D	GTA
H	Q	Y	Y	F	D	G	N	T	S		V
TCA	ACA	TAT	CTT	GAT	GCC	TCA	TAC	TTT	AAA	AAG	CTG
S	T	Y	L	D	A	S	Y	F	K	K	L
CAA	CAG	CTG	TTT	GAT	AAA	GTG	AAA	gat	GTT	TTG	AAA
Q	Q	L	F	D	K	V	K	D	V	L	K
AAT	TCT	CCA	CAT	AAA	GAC	AAA	CCA	TTA	TGG W	CTT	GGA
N	S	P	H	K	D	K	P .	L		L	G
GAG	ACA	AGT	TCT	GGA	TGC	AAC	AGC	GGC	ACA	AAA	GAT
E	T	S	S	G	Y	N	S	G	T	K	D
GTA	TCC	GAT	CGA	TAT	GTT	TCA	GGA	TTT	CTA	ACA	TTA
V	S	D	R	Y	V	S	G	F	L	T	L
GAC	AAG	TTG	GGT	CTC	AGT	GCA	GCC	AAC	AAT	GTA	AAG
D	K	L	G	L	S	A	A	N		V	K
GTT	GTT	ATA	AGA	CAG	ACA	ATA	TAC	AAT	GGA	TAT	TAT
V	V	I	R	Q	T	I	Y	N	G	Y	Y
GGT	CTC	CTT	GAT	AAA	AAC	ACT	TTA	GAG	CCA	AAT	CCT
G	L	L	D	K	N	T	L	E	P	N	P
GAT	TAC	TGG	TTA	ATG	CAT	GTT	CAC	AAT	TCT	TTG	GTC
D	Y	W	L	M	H	V	H	N	S	L	V
GGA G	AAT N	ACA T	GTT V	TTT F	AAA K	G <b>T</b> T V		GTT V	GGT G		CCA P
ACT	AAT	AAA	ACG	AGA	GTC	TAT	GCA	CAA	TGT	ACC	AAG
T	N	K	T	R	V	Y	A	Q	C	T	K
ACA	AAT	AGC	AAA	CAC	ACT	CAA	GGC	AAG	TAT	TAC	AAG
T	N	S	K	H	T	Q	G	K	Y	Y	K
GGC G	TCT S	_	ACA T	ATC I	TTT F	GCA A	CTT L	AAT N	GTT V	GGA	GAT D

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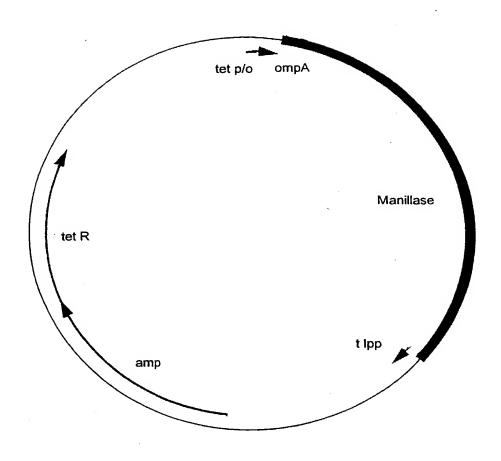
#### Fig 10 (contnd)

GAA	GAA	GTA	ACG	TTA	AAG	ATC	GAT	CAA	TAC	GGC	GGT
E	E	V	T	L	K	I	D	Q	Y	G	G
AAA	AAA	ATT	TAT	TCA	TAC	ATT	CTG	ACA	CCT	GAA	GGA
K	K	I	Y	S	Y	I	L	T	P	E	G
GGA	CAA	CTT	ACA	TCA	CAG	AAA	GTT	CTC	TTG	AAT	GGA
G	Q	L	T	S	Q	K	V	L	L	N	G
AAG	GAA	TTG	AAC	TTA	GTG	TCT	GAT	CAG	TTA	CCA	GAA
K	E	L	N	L	V	S	D	Q	L	P	E
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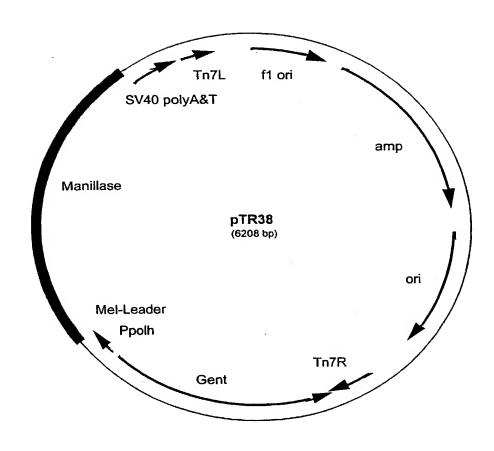
Fig. 11:



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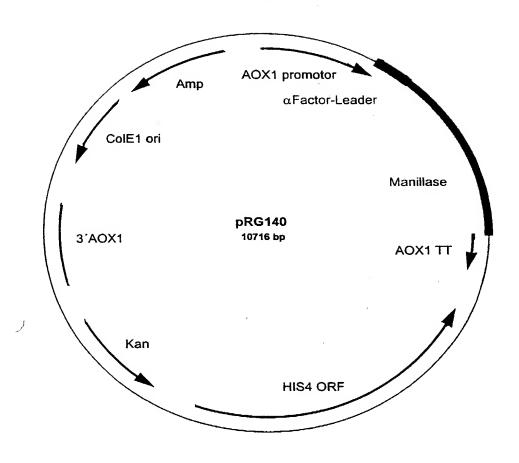
Fig. 12:



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Fig. 13:



Docket N	10.
MERCK	

# Declaration and Power of Attorney For Patent Application

**English Language Declaration** As a below named inventor, I hereby declare that: My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled Hyaluronidase from the Hirudinaria mallinensis, isolation, purification and recombinant method of production the specification of which (check one) ☐ is attached hereto. as United States Application No. or PCT International mas filed on Application Number and was amended on (if applicable) I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56. I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed. Priority Not Claimed Prior Foreign Application(s) 12.06.99 99111468.7 EΡ (Day/Month/Year Filed) (Country) (Number) (Day/Month/Year Filed) (Country) (Number) (Day/Month/Year Filed) (Country) (Number)

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ection 365(c) of any PCT Internation as the subject matter of Inited States or PCT Internation I.S.C. Section 112. I acknowled office all information known to a	ational application designating each of the claims of this apair all application in the manner lige the duty to disclose to the me to be material to patental able between the filing date contains.	of any United States application(s), ag the United States, listed below a pplication is not disclosed in the provided by the first paragraph of a United States Patent and Trademability as defined in Title 37, C. F. of the prior application and the nation
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ection 365(c) of any PCT Intern sofar as the subject matter of inited States or PCT Internation I.S.C. Section 112. I acknowled office all information known to ection 1.56 which became availa	ational application designating each of the claims of this apair all application in the manner lige the duty to disclose to the me to be material to patental able between the filing date contains.	ng the United States, listed below a pplication is not disclosed in the pure provided by the first paragraph of the United States Patent and Tradem ability as defined in Title 37, C. F.
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fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

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Third inventor's signature X live High an	1 2. OK¶ate2001
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Fourth inventor's signature Parl	1.7 OKI 2001
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	Date
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#### SEQUENCE LISTING

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ac Th 38	r As	t ag n Se	c aaa r Ly:	a cat s His	act Thr 390	Gln	agc Ser	aga Arg	tat Tyr	tac Tyr 395	aag Lys	ggc	tct Ser	ttg Leu	aca Thr 400	1200
at Il	c tt e Ph	t gc e Al	a ct a Le	t aat u Asn 405	l Val	gga Gly	gat Asp	gaa Glu	gat Asp 410	gta Val	acg Thr	tta Leu	aag Lys	atc Ile 415	GLY	1248
ca Gl	a ta n Ty	c ag r Se	c gg r Gl 42	y Lys	a aaa s Lys	a att s Ile	tat Tyr	tca Ser 425	Tyr	att	ctg Leu	aca Thr	cct Pro 430	gaa Glu	gga Gly	1296
G]	a ca y Gl	a ct n Le	eu Th	a tca ir Se	a caq r Gli	g aaa n Lys	gtt Val	Leu	ttg Lev	aat Asr	gga Gly	aag Lys 445	gaa Glu	tto	g aac n Asn	1344
tt Le	eu Xa	g to aa Se 50	ct ga er As	at ca sp Gl:	g tta n Le	a cca u Pro 455	Glr	r cta	a aat a Asr	gca n Ala	a gat a Asp 460	Giu	tcc Ser	aaa Lys	a aca s Thr	1392
S	et ti er Pl	tc a	cc tt hr Le	a tc eu Se	c cc r Pr 47	o Ly:	g aca s Thi	a ttt r Phe	ggt Gl	t tt1 y Phe 47	e Pne	gtt Val	gtt Val	tc. Se:	c gat r Asp 480	1440
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	Ala 65	As	n A	Arg	Leu	Phe	Phe 70	Asp	Leu	Asp	Glu	Asn 75	Asn	Lys	Trp	Lys	Asp 80
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	Arg	Tr	pΙ	Leu	Phe 100	Arg	Lys	Gln	Asn	Asn 105	Leu	Lys	Lys	Glu	Thr 110	Phe	Asp
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	Ser	L	/S	Gly	Tyr	Gly 165	Asp	Asn	Ile	Asp	170	Glu	Leu	Gly	Asn	Gly 175	Pro
	Asp	р H:	is	Thr	Ser 180		His	Asn	Leu	Thr 185	Glu	ı Lys	Gln	. Val	. Gly 190	Glu	Asp
	Phe	e L	ys	Ala 195	Leu	His	Lys	Val	Leu 200	Glu	Lys	з Туг	Pro	205	Leu	Asn	Lys
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	Ly 22	_	ly	Leu	Ala	Asp	Glu 230		a Gly	Ası	o His	s Val 23	l Thi	Ala	a Phe	Thi	Leu 240
	Hi	s G	ln	Туг	туг	Phe 245		Gly	y Asn	Th	25	r Ası	o Vai	l Se	r Ile	255	Leu
	As	рΑ	la	Thr	Ty:		e Lys	Ly	s Lev	1 Gl: 26	n Gl	n Le	u Ph	e As	p Lys 270	s Vai	L Lys
	As	p V	al	Let 275		s Ası	o Ser	Pr	o His	s Ly	s As	p Ly	s Pr	o Le 28	u Trj 5	p Le	ı Gly

Glu Thr Ser Ser Gly Tyr Asn Ser Gly Thr Glu Asp Val Ser Asp Arg

295

290

300

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Tyr 305	Val	Ser	Gly	Phe	Leu 310	Thr	Leu	Asp	Lys	Leu 315	Glý	Leu	Ser	Ala	Ala 320	
Asn	Asn	Val	Lys	Val 325	Val	Ile	Arg	Gln	Thr 330	Ile	Tyr	Ser	Gly	Tyr 335	Tyr	î
Gly	Pro	Leu	Asp 340	Lys	Asn	Thr	Leu	Glu 345	Pro	Asn	Pro	Asp	Tyr 350	Trp	Leu	0
Met	His	Val 355	His	Asn	Ser	Leu	Val 360	Gly	Asn	Thr	Val	Phe 365	Lys	Val	Asp	
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agt Sei	gaq Gli	g tct ı Sei	tto Phe	e His	gga Gly	gtt Val	gcc Ala	ttt Phe 25	Asp	gcg Ala	g tot ser	cta Lev	tto Phe 30	e Ser	ccc Pro	96
aaq Ly:	g gg	t cci y Pro 3!	o Trp	g ago Sei	ttt Phe	gtt Val	aat L Asr 40	ılje	aco Thi	tct Ser	c cca	a aaa b Lys 45	E Lev	g tto ı Phe	aaa Lys	144
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gcc Ala 65	Ası	t to	g c	tg 1	Phe !	Phe 1	gac Asp	ttg ( Leu i	gac ( Asp (	gaa Glu	aat Asn 75	aat Asn	aag † Lys '	tgg Trp	aag Lys	gat Asp 80	240
tat Tyr	tg:	g go Al	ct t La E	tt ?he	aaa Lys 85	gac Asp	aaa Lys	acc Thr	ecc Pro	gaa Glu 90	act Thr	gcg Ala	aca Thr	ata Ile	aca Thr 95	agg Arg	288
aga	tg Tr	g ct p Le	eu F	tc Phe	aga Arg	aaa Lys	caa Gln	aat Asn	aat Asn 105	ctg Leu	aaa Lys	aag Lys	gag Glu	act Thr 110	ttt Phe	gac Asp	336
ga: As:	t tt p Le	ag uV	al I	aaa Lys	cta Leu	aca Thr	aag Lys	gga Gly 120	agc Ser	aag Lys	atg Met	aga Arg	ttg Leu 125	tta Leu	ttc Phe	gat Asp	384
tt Le	g aa u As 13	n A	cc (	gaa Glu	gtg Val	agg Arg	act Thr 135	ggt Gly	tat Tyr	gaa Glu	att Ile	gga Gly 140	aag Lys	aag Lys	acg Thr	aca Thr	432
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ti Pi	ic a	ys A	jca Ala 195	ctg Leu	cat His	aaa Lys	gtt Val	tta Leu 200	gag Glu	aaa Lys	tat Tyr	cca Pro	act Thr 205	ctt Leu	aac Asi	aag Lys	624
g G	ly S	cg o er 1	ecc Pro	gtt Val	ggt Gly	cca Pro	gat Asp 215	) Val	ggğ	tgg Trp	ato Met	ggc : Gly 220	, var	ago	tac Ty:	c gtc r Val	672
L	ag g ys G 25	ga ly	ttg Leu	gca Ala	gac Asp	ggg Gly 230	Ala	ggt Gly	gac Asp	ctt Lev	gta Val 235	LTni	gct Ala	ttt Phe	aca e Th	a cta r Leu 240	720
С	ac c is G	aa In	tat Tyr	tat Tyr	ttc Phe 245	Asp	gga Gly	a aac y Asr	acc Thr	tct Sei 250	c As	t gta p Val	a tca l Ser	a aca	a ta r Ty 25	t ctt r Leu 5	768
Ç	at o	)la	tca Ser	tac Tyr 260	Phe	aaa Lys	aaq Ly	g cto s Lei	g caa 1 Glr 265	i Gli	g cto n Le	g tt u Ph	t gat e Asp	aa b Ly 27	s va	g aaa l Lys	816
Ģ	gat ( Asp '	gtt Val	ttg Leu 275	Ly	a aat s Asr	tct n. Sei	cc Pro	a cat o Hi: 280	s Ly:	a ga s As	c aa p Ly	a cc s Pr	a tta o Lei 28	u Tr	g ct p Le	t gga u Gly	864
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	tat Tyr 305	gtt Val	tca Ser	gg Gl	a tt <sup>i</sup> y Pho	t cta e Le	u Th	a tt r Le	a ga u As	c aa p Ly	g tt s Le 31	eu Gi	t ct y Le	c aç u Se	gt go	ca gco la Ala 320	<u>.</u>

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tc Se:	r Phe	e acc	tt: r Le	a tcc u Ser	2 CC2	Ly:	g aca	a tti r Phe	t ggt e Gl	t tti y Phe 47	e Pne	gtt Val	gt: L Va.	t tco l Sei	gat Asp 480	1440
gc Al	t aai a Asi	t gt n Va	t ga l Gl	a gca u Ala 485	а Суз	y aa: s Ly:	r aa: s Ly:	r s					0		•	1464
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Туг	Trp	Ala	Phe	Lys 85	Asp	Lys	Thr	Pro	Glu 90	Thr	Ala	Thr	Ile	Thr. 95	Arg
Arg	Trp	Leu	Phe 100	Arg	Lys	Gln	Asn	Asn 105	Leu	Lys	Lys	Glu	Thr 110	Phe	Asp
Asp	Leu	Val 115	Lys	Leu	Thr	Lys	Gly 120	Ser	Lys	Met	Arg	Leu 125	Leu	Phe	Asp
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His	Gln	Tyr	Tyr	Phe 245	Asp	Gly	Asn	Thr	Ser 250	Asp	Val	Ser	Thr	Tyr 255	Leu
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Gl	Leu	ı Lev	Asp 340		Asn	Thr	Leu	Glu 345		) Asn	Pro	Asp	Tyr 350		Leu
Met	. His	val 355		Asn	Ser	Leu	Val 360		Asn	Thr	Val	Phe 365		: Val	Asp
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	Gly	Gln	Leu 435	Thr	Ser	Gln	Lys	Val 440	Leu	Leu	Asn	Gly	Lys 445	Glu	Leu	Asn		
	_			7	C1 n	T 011	Dwa		7 011	· · ·	- נת	. 7		C	7	т. тъ-		
	Leu	450	Ser	Asp.	GTII	пец	455	G_U	rea	ASII	HIA	460	GIU	ser	ьys	inr	130	
•	Ser 465	Phe	Thr	Leù	Ser	Pro 470	Lys	Thr	Phe	Gly	Phe 475	Phe	Val	Val	Ser	Asp 480		
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Caccu	reged adaptages of objection	*				٠,
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